Novel therapeutic compositions for treating infection by *Lawsonia spp.*

RELATED APPLICATION DATA

This application claims benefit of priority from Australian Patent Application No. PR1381 filed on November 10, 2000, and from United States Patent Application Serial No. 60/249596 filed on November 17, 2000.

FIELD OF THE INVENTION

The present invention relates generally to therapeutic compositions for the treatment and/or prophylaxis of intestinal disease conditions in animals and birds caused or exacerbated by *Lawsonia intracellularis* or similar or otherwise related microorganism. In particular, the present invention provides a novel gene derived from *L. intracellularis* which encodes an immunogenic polypeptide. The polypeptide described herein, selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue, analogue or derivative of any one or more of said polypeptides, is particularly useful as an antigen in vaccine preparation for conferring humoral immunity against *L. intracellularis* and related pathogens in animal hosts. The present invention is also directed to methods for the treatment and/or prophylaxis of such intestinal disease conditions and to diagnostic agents and procedures for detecting *L. intracellularis* or similar or otherwise related microorganisms.

GENERAL

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. All patents, patent applications, and publications cited herein are incorporated by reference in their entirety.

Reference hereinafter to *"Lawsonia intracellularis"* or its abbreviation *"L. intracellularis"* includes all microorganisms similar to or otherwise related to this microorganism, as described by Stills (1991) or Jones *et al.*(1997) or Lawson *et al.* (1993) or McOrist *et al.* (1995).

References herein to "AGAL" shall be taken to mean a reference to the Australian Government Analytical Laboratories located at 1 Suakin Street, Pymble, New South Wales 2073, Australia. All biological deposits referred to herein in respect of the plasmids assigned AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); and NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN) have been made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

As used herein, the word "*flhB*", or the term "*flhB* gene", shall be taken to refer to a gene encoding the antigenic flhB polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 1 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#2 which has been deposited under AGAL Accession No. NM00/16477. The word *"flhB"* or the term "*flhB* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 1 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmidpGTE#2 which has been deposited under AGAL Accession No. NM00/16477. It shall also be understood that the term "flhB polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 2 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#2 which has been deposited under AGAL Accession No. NM00/16477. The term "flhB polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 2 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#2 which has been deposited under AGAL Accession No. NM00/16477.

As used herein, the word "*fliR*", or the term "*fliR* gene", shall be taken to refer to a

gene encoding the antigenic fliR polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 3 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#3 which has been deposited under AGAL Accession No.NM00/16478. The word *"fliR"* or the term "*fliR* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 3, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#3 which has been deposited under AGAL Accession No.NM00/16478. It shall also be understood that the term "fliR polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 4 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#3 which has been deposited under AGAL Accession No.NM00/16478. The term "fliR polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 4 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#3 which has been deposited under AGAL Accession No.NM00/16478.

As used herein, the word "*ntrC*", or the term "*ntrC* gene", shall be taken to refer to a gene encoding the antigenic ntrC polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 5 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#6 which has been deposited under AGAL Accession No.NM00/16481. The word *"ntrC"* or the term "*ntrC* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 5, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#6 which has been deposited under AGAL Accession No.NM00/16481. It shall also be understood that the term "ntrC polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 6 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#6 which has been deposited under AGAL Accession No.NM00/16481. The term "ntrC polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically

cross-reactive with the polypeptide of SEQ ID NO: 6 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#6 which has been deposited under AGAL Accession No.NM00/16481.

As used herein, the word "*glnH*", or the term "*glnH* gene", shall be taken to refer to a gene encoding the antigenic glnH polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 7 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#1 which has been deposited under AGAL Accession No.NM00/16476. The word *"glnH"* or the term "*glnH* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 7, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#1 which has been deposited under AGAL Accession No.NM00/16476. It shall also be understood that the term "glnH polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 8 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#1 which has been deposited under AGAL Accession No.NM00/16476. The term "glnH polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 8 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#1 which has been deposited under AGAL Accession No.NM00/16476.

As used herein, the word "*motA*", or the term "*motA* gene", shall be taken to refer to a gene encoding the antigenic motA polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 9, or to the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and which has homology to SEQ ID NO: 9. The word *"motA"* or the term "*motA* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 9, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and

which has homology to SEQ ID NO: 9. It shall also be understood that the term "motA polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 10 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and which has homology to SEQ ID NO: 9. The term "motA polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 10 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and having homology to SEQ ID NO: 9.

As used herein, the word "*motB*", or the term "*motB* gene", shall be taken to refer to a gene encoding the antigenic motB polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 11 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and having homology to SEQ ID NO: 11. The word *"motB"* or the term "*motB* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 11, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and having homology to SEQ ID NO: 11. It shall also be understood that the term "motB polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 12 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and having homology to SEQ ID NO: 11. The term "motB polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 12 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#4 which has been deposited under AGAL Accession No.NM00/16479 and having homology to SEQ ID NO: 11.

As used herein, the word "*tlyC*", or the term "*tlyC* gene", shall be taken to refer to a gene encoding the antigenic tlyC polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 13 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#5 which has been deposited under AGAL Accession No.NM00/16480. The word *"tlyC"* or the term "*tlyC* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 13, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#5 which has been deposited under AGAL Accession No.NM00/16480. It shall also be understood that the term "tlyC polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 14 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#5 which has been deposited under AGAL Accession No.NM00/16480. The term "tlyC polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 14 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#5 which has been deposited under AGAL Accession No.NM00/16480.

As used herein, the word "*ytfM*", or the term "*ytfM* gene", shall be taken to refer to a gene encoding the antigenic ytfM polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 15 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#7 which has been deposited under AGAL Accession No.NM00/16482. The word *"ytfM"* or the term "*ytfM* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 15, or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#7 which has been deposited under AGAL Accession No.NM00/16482. It shall also be understood that the term "ytfM polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 16 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#7 which has been deposited under AGAL Accession No.NM00/16482. The term "ytfM polypeptide" shall further

be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 16 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#7 which has been deposited under AGAL Accession No.NM00/16482.

As used herein, the word "*ytfN*", or the term "*ytfN* gene", shall be taken to refer to a gene encoding the antigenic ytfN polypeptide of the present invention, which gene comprises the nucleotide sequence set forth in SEQ ID NO: 17 or the nucleotide sequence of the *L. intracellularis* gene contained in the plasmid pGTE#8 which has been deposited under AGAL Accession No. NM01/23286. The word *"ytfN"* or the term "*ytfN* gene" shall further be taken to include a degenerate or complementary nucleotide sequence to SEQ ID NO: 17 or the nucleotide sequence of the L. intracellularis gene contained in the plasmid pGTE#8 which has been deposited under AGAL Accession No. NM01/23286. It shall also be understood that the term "ytfN polypeptide" refers to a polypeptide of the invention which comprises the amino acid sequence set forth in SEQ ID NO: 18 or a polypeptide encoded by the *L. intracellularis* gene contained in the plasmid pGTE#8 which has been deposited under AGAL Accession No. NM01/23286. The term "ytfN polypeptide" shall further be taken to include a polypeptide which is functionally-related to or immunologically cross-reactive with the polypeptide of SEQ ID NO: 18 or a polypeptide encoded by the L. intracellularis gene contained in the plasmid pGTE#8 which has been deposited under AGAL Accession No. NM01/23286.

As used herein the words "from" or "of", and the term "derived from" shall be taken to indicate that a specified product, in particular a macromolecule such as a polypeptide, protein, gene or nucleic acid molecule, antibody molecule, Ig fraction, or other macromolecule, or a biological sample comprising said macromolecule, may be obtained from a particular source, organism, tissue, organ or cell, albeit not necessarily directly from that source, organism, tissue, organ or cell.

Throughout this specification, unless the context requires otherwise, the word

"comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps, features, compositions and compounds.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

BACKGROUND OF THE INVENTION

The meat-producing sector of the agricultural industry is dependent upon the health of its livestock and there is a need to maintain disease-free livestock for human consumption. The industry is subject to rapid economic downturn in response to disease conditions adversely affecting livestock and the quality of meat products derived therefrom, including those diseases which may potentially be transmitted to humans. It is important, therefore, to have well defined treatments and prophylactic and diagnostic procedures available to deal with infections or potential infections in livestock animals and humans.

Meat products derived from porcine and avian species are significant commercial products in the agriculture industry. In particular, pigs form a major component of the meat industry. However, pigs are sensitive to a wide spectrum of intestinal diseases

collectively referred to as porcine proliferative enteropathy (PPE). These diseases have previously been known as intestinal adenomatosis complex (Barker and van Drumel, 1985), porcine intestinal adenomatosis (PIA), necrotic enteritis (Rowland and Lawson, 1976), proliferative haemorrhagic enteropathy (Love and Love, 1977), regional ileitis (Jonsson and Martinsson, 1976), haemorrhagic bowel syndrome (O'Neil, 1970), porcine proliferative enteritis and *Campylobacter* spp – induced enteritis (Straw, 1990).

There are two main forms of PPE: a non-haemorrhagic form represented by intestinal adenomatosis which frequently causes growth retardation and mild diarrhoea; and a haemorrhagic form, which is often fatal, represented by proliferative haemorrhagic enteropathy (PHE), where the distal small intestine lumen becomes engorged with blood. PPE has been reported in a number of animal species including pigs (McOrist *et al*, 1993), hamsters (Stills, 1991), ferrets (Fox *et al*, 1989), guinea pigs (Elwell *et al*, 1981), rabbits (Schodeb and Fox, 1990) as well as avian species (Mason *et al*, 1998).

PPE is a significant cost component associated with the pig industry, especially in terms of stock losses, medication costs, reduced growth rates of pigs and increased feed costs. PPE also contributes to downstream indirect costs in, for example, additional labour costs and environmental costs in dealing with antibiotic residue contamination, and in control measures to prevent the organism from being passed on or carried to other animals or humans.

L. intracellularis is a causative agent of PPE (McOrist *et al*, 1995). *L. intracellularis* is an intracellular, possibly obligate intracellular, bacterium. It can only be cultured *in vitro* with tissue culture cells (Jones *et al.*, 1997; Lawson *et al.*, 1993; McOrist *et al*, 1995; International Patent Application No. PCT/US96/09576). *L. intracellularis* is located in the cytoplasm of the villus cells and intestinal crypt cells of infected animals. Pigs suffering from PPE are characterised by irregularities in the villus cells and intestinal crypt structure with epithelial cell dysplasia, wherein crypt abscesses

form as the villi and intestinal crypts become branched and fill with inflammatory cells.

Current control strategies for PPE rely on the use of antibacterials. However, such a strategy is considered to only be short to medium term, especially since governmental regulatory pressures tend to discourage animal husbandry practices which involve the use of prophylactic antibiotics. There is a need, therefore, to develop effective, safe and low cost alternatives to the use of antibiotics and, in particular, to develop vaccine preparations capable of conferring protective immunity against *L. intracellularis* infection in livestock animals.

The most effective vaccine preparations are generally comprised of a highly antigenic component, such as a polypeptide or other macromolecule which is derived from the pathogenic organism against which the vaccine is directed, wherein said antigenic component produces little or no contraindications when administered to a susceptible host animal, and produces little or no antigenic cross-reactivity with desirable organisms, such as non-pathogenic organisms that are a part of the normal flora of the intestinal tract or other tissues of said host animal. In summary, an effective vaccine preparation must be immunogenic, specific and safe.

Accordingly, there is a need to identify highly immunogenic antigens produced by the bacterium *L. intracellularis*.

International Patent Application No. PCT/AU96/00767 describes several *L. intracellularis* partial genetic sequences, and partial polypeptides encoded thereby. However, there is a need to further identify polypeptide immunogens produced by the bacterium *L. intracellularis* and immunogenic peptides derived therefrom, including those immunogens which are genus- or species-specific, for use in improved vaccine compositions. The presently-described invention provides such immunogens.

SUMMARY OF THE INVENTION

One aspect of the present invention is directed to an isolated or recombinant immunogenic polypeptide which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of a polypeptide derived from *Lawsonia spp,* in particular a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue, analogue or derivative of any one or more of said polypeptides.

Preferably, the isolated or recombinant immunogenic polypeptide is selected from the group consisting of the following:

(i) a polypeptide which comprises an amino acid sequence which has at least about 60% sequence identity overall to an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18;

(ii) a polypeptide which comprises an amino acid sequence which has at least about 60% sequence identity overall to an amino acid sequence encoded by *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a polypeptide which comprises at least about 5 contiguous amino acids of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18;

(iv) a polypeptide which comprises at least about 5 contiguous amino acids of an amino acid sequence encoded by *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN); and

(v) a homologue, analogue or derivative of any one of (i) to (iv) which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

In an alternative preferred embodiment, the isolated or recombinant immunogenic polypeptide is selected from the group consisting of the following:

(i) a polypeptide which comprises an amino acid sequence encoded by a nucleotide sequence having at least about 60% sequence identity overall to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(ii) a polypeptide which comprises an amino acid sequence encoded by a nucleotide sequence having at least about 60% sequence identity overall to the nucleotide sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a polypeptide encoded by at least about 15 contiguous nucleotides of a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(iv) a polypeptide encoded by at least about 15 contiguous nucleotides of a nucleotide sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN); and

(v) a homologue, analogue or derivative of any one of (i) to (iv) which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

In a particularly preferred embodiment, the polypeptide of the present invention

comprises or consists of an amino acid sequence selected from the group consisting of:

(i) an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18; and

(ii) an amino acid sequence encoded by *L. intracellularis* DNA contained within a deposited plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN).

A further aspect of the present invention provides a vaccine composition for the prophylaxis or treatment of infection in an animal, such as a pig or bird, by *L. intracellularis* or a similar or otherwise related microorganism, said vaccine composition comprising an immunologically effective amount of an immunogenic component which comprises an isolated or recombinant polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides as described herein and one or more carriers, diluents and/or adjuvants suitable for veterinary or pharmaceutical use.

A further aspect of the invention extends to an immunologically interactive molecule, such as an antibody or antibody fragment, which is capable of binding to an immunogenic polypeptide of the invention selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides.

A further aspect of the invention provides a method of diagnosing infection of an animal by *L. intracellularis* or a related microorganism, said method comprising the steps of contacting a biological sample derived from said animal with an immunologically interactive molecule of the present invention for a time and under conditions sufficient for a complex, such as an antigen:antibody complex, to form,

and then detecting said complex formation.

A further aspect of the present invention contemplates a method of determining whether or not an animal has suffered from a past infection, or is currently infected, by *L. intracellularis* or a related microorganism, said method comprising contacting a tissue or fluid sample, such as blood or serum derived from said animal, with an immunogenic polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a peptide derived therefrom, for a time and under conditions sufficient for a complex, such as an antigen:antibody complex, to form, and then detecting said complex formation.

A further aspect of the present invention provides an isolated nucleic acid molecule which comprises a sequence of nucleotides that encodes, or is complementary to a nucleic acid molecule that encodes, a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, including any and all genes selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes as defined hereinabove.

In a preferred embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a polypeptide that is immunologically cross-reactive with *L. intracellularis* or other causative agent of PPE, wherein said nucleotide sequence is selected from the group consisting of:

(i) a nucleotide sequence having at least about 60% sequence identity overall to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(ii) a nucleotide sequence having at least about 60% sequence identity overall to *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7

ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a nucleotide sequence which comprises at least about 15 contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(iv) a nucleotide sequence which comprises at least about 15 contiguous nucleotides of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(v) a nucleotide sequence which hybridizes under at least low stringency, more preferably moderate stringency, and most preferably high stringency conditions to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17 or a complementary nucleotide sequence thereto;

(vi) a nucleotide sequence which hybridizes under at least low stringency, more preferably moderate stringency, and most preferably high stringency conditions to *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN); and

(vii) a homologue, analogue or derivative of any one of (i) to (vi) which encodes a polypeptide which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

In a particularly preferred embodiment, the isolated nucleic acid molecule of the present invention comprises or consists of a nucleotide sequence selected from the group consisting of:

(i) a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1,3, 5, 7, 9, 11, 13, 15, and 17; or a degenerate variant thereof;

(ii) a nucleotide sequence of the *L. intracellularis* DNA contained within a deposited plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN); and

(iii) a nucleotide sequence that encodes the same polypeptide as (i) or (ii), wherein said polypeptide is selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN;

(iv) a nucleotide sequence that is complementary to (i) or (ii) or (iii); and

(v) a nucleotide sequence that hybridises under high stringency conditions to the complement of a sequence selected from the group consisting of: SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15 and 17, wherein said nucleotide sequence is the complement of a sequence that encodes a polypeptide that is immunologically cross-reactive to a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN.

A still further aspect of the invention provides a diagnostic method of detecting *L. intracellularis* or related microorganism in a biological sample derived from an animal subject, said method comprising the steps of hybridising one or more polynucleotide or oligonucleotide probes or primers derived from a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes, or a homologue, analogue or derivative thereof, to said sample, and then detecting said hybridisation using a detection means. The detection means according to this aspect of the invention is any nucleic acid-based hybridisation or amplification reaction.

A further aspect of the invention provides an isolated probe or primer derived from a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC,*

ytfM, and *ytfN* genes. In a particularly preferred embodiment, the probe or primer of the invention is useful for isolating the *ytfM* and/or *ytfN* genes described herein. More preferably, the probe or primer of the invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 19 to SEQ ID NO: 68 or a complementary nucleotide sequence thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a photographic representation showing expression of recombinant YtfN protein. The 5' portion of the gene up to the BglII site was cloned into pET-30a. A plasmid with the fragment inserted in the proper orientation was transformed into E. coli BL21 (DE3) cells, and a single clone was propagated. Induction was at OD_{625}=2.9 with 0.1 mM IPTG. Lane 1, whole cell lysate (WCL) from uninduced cells; lanes 2 and 3, WCL at 2.25 and 3 hrs post-induction, respectively. Arrow indicates the position of recombinant YtfN protein.

DETAILED DESCRIPTION OF THE INVENTION

In work leading up to the present invention, the inventors sought to identify immunogenic proteins of *L. intracellularis* for use in vaccines for the prophylaxis and treatment of PPE in animals, including pigs and birds.

Accordingly, one aspect of the present invention is directed to an isolated or recombinant immunogenic polypeptide which comprises, mimics or cross-reacts with a B-cell or T-cell epitope of a polypeptide derived from *Lawsonia spp,* selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN, or a homologue, analogue or derivative of any one or more of said polypeptides.

Epitopes of *Lawsonia spp*. may be B cell epitopes or T-cell epitopes. It is well-known that antibody-binding sites (B-cell epitopes) involve linear as well as conformational epitopes (van Regenmortel, 1992). B-cell epitopes are predominantly conformational. In contrast, T-cells recognize predominantly linear epitope sequences in combination with MHC class II molecules.

A precise identification and careful selection of epitopes of *Lawsonia spp.* facilitates the development of diagnostic reagents and vaccine compositions for the effective treatment or prophylaxis of *Lawsonia* infections. Epitope identification and characterization (i.e., determination of the molecular weight, amino acid sequence, and structure of epitopes of *Lawsonia spp.*) may be performed using art-recognised techniques. For the detection of conformational epitopes, degrading and denaturing of the epitope molecule must be avoided in order to conserve the three-dimensional structure, because the antigen-antibody reaction will be diminished if the secondary structure of the epitope is altered significantly. In practice, the characterisation and isolation of linear non-conformational epitopes is easier, because any immunoreactive regions are contained within a single polypeptide or peptide fragment which is capable of being purified under a range of conditions.

Both non-conformational and conformational epitopes may be identified by virtue of their ability to bind detectable amounts of antibodies (such as IgM or IgG) from sera of animals immunised against or infected with *Lawsonia spp.* and, in particular *L. intracellularis,* or an isolated polypeptide derived therefrom or, alternatively, by virtue of their ability to bind detectable amounts of antibodies in a purified Ig fraction derived from such sera. The antibodies may be derived from or contained within pools of polyclonal sera, or may be monoclonal antibodies. Antibody fragments or recombinant antibodies, such as those expressed on the surface of a bacteriophage or virus particle, such as in a phage display library, may also be employed.

The determination of T-cell epitopes is performed by analysing the ability of the epitope peptides to induce the proliferation of peripheral blood lymphocytes or T-cell clones. The identification of T-cell epitopes is accomplished using a variety of methods as known in the art, including the use of whole and fragmented native or recombinant antigenic protein, as well as the more commonly employed "overlapping peptide" method. In the latter method, overlapping peptides which span the entire sequence of a polypeptide derived from *Lawsonia spp.* are synthesized and tested

for their capacity to stimulate T-cell cytotoxic or proliferative responses *in vitro*.

Structure determination of both conformational non-linear and non-conformational linear epitopes may be performed by nuclear magnetic resonance spectroscopy (NMR) and X-ray crystallographic analysis. The determination of epitopes using X-ray techniques requires the protein-antibody complex to be crystallized, whereas NMR allows analysis of the complex in a liquid state. NMR measures the amount of amino acids as well as the neighbourhood of protons of different amino acid residues, wherein the alternating effect of two protons along the carbon backbone is characteristic of a particular epitope.

A successful method to recognize non-conformational linear epitopes is the immunoblot and in particular, the Western blot. Peptides may be generated from a complete *Lawsonia spp*. polypeptide by digestion with site-specific proteases, such as trypsin or chymotrypsin, and the peptides generated thereby can be separated using standard electrophoretic or chromatographic procedures. For example, after electrophoresis according to molecular weight using SDS/PAGE (SDS/polyacrylamide gel electrophoresis) and/or according to isoelectric point using IEF (isoelectric focussing) or alternatively, by two-dimensional electrophoresis, the peptides can be transferred to immobilizing nylon or nitrocellulose membranes and incubated with sera raised against the intact polypeptides. Peptides that comprise immunogenic regions (i.e., B-cell or T-cell epitopes) are bound by the antibodies in the sera and the bound antibodies may be detected using secondary antibodies, such as anti-IgG antibodies, that have been labelled radioactively or enzymatically. The epitopes may then be characterised by purification based upon their size, charge or ability to bind specifically to antibodies against the intact polypeptide, using one or more techniques, such as size-exclusion chromatography, ion-exchange chromatography, affinity chromatography or ELISA among others. After purification of the epitope, only one band or spot should be detectable with gel electrophoresis. The N-terminal or total sequencing of the polypeptide or peptide fragment offers the possibility to compare the amino acid sequence with known proteins in databases.

Several computer-driven algorithms have now been devised to search for T-cell epitopes in proteins (Margalit *et al*, 1987; Vajda and C. DeLisi, 1990; Altuvia *et al.*, 1994; Parker *et al.*1994; DeGroot *et al.*,1995; Gabriel *et al*, 1995; Meister *et al.*, 1995). These algorithms search the amino acid sequence of a given protein for characteristics believed to be common to immunogenic peptides, locating regions that are likely to induce a cellular immune response *in vitro*. Computer-driven algorithms can identify regions of a *Lawsonia spp.* polypeptide that contain epitopes and are less variable among different isolates. Alternatively, computer-driven algorithms can rapidly identify regions of each isolate's more variable proteins that should be included in a multivalent vaccine.

The AMPHI algorithm (Margalit *et al.*,1987), which is based on the periodicity of T cell epitopes, has been widely used for the prediction of T-cell antigenic sites from sequence information alone. Essentially, AMPHI describes a common structural pattern of MHC binding motifs, since MHC binding motifs (i.e., patterns of amino acids that appear to be common to most of the peptides that bind to a specific MHC molecule) appear to exhibit the same periodicity as an alpha helix. Identification of T-cell epitopes by locating MHC binding motifs in an amino acid sequence provides an effective means of identifying immunogenic epitopes in diagnostic assays.

The EpiMer algorithm (Meister *et al.*, 1995; Gabriel *et al.*, 1995; DeGroot *et al.*, 1995) locates clustered MHC binding motifs in amino acid sequences of proteins, based upon the correlation between MHC binding motif-dense regions and peptides that may have the capacity to bind to a variety of MHC molecules (promiscuous or multi-determinant binders) and to stimulate an immune response in these various MHC contexts as well (promiscuous or multi-determinant epitopes). The EpiMer algorithm uses a library of MHC binding motifs for multiple class I and class II HLA alleles to predict antigenic sites within a protein that have the potential to induce an immune response in subjects with a variety of genetic backgrounds. EpiMer locates matches to each MHC-binding motif within the primary sequence of a given protein

antigen. The relative density of these motif matches is determined along the length of the antigen, resulting in the generation of a motif-density histogram. Finally, the algorithm identifies protein regions in this histogram with a motif match density above an algorithm-defined cutoff density value, and produces a list of subsequences representing these clustered, or motif-rich regions. The regions selected by EpiMer may be more likely to act as multi-determinant binding peptides than randomly chosen peptides from the same antigen, due to their concentration of MHC-binding motif matches. The selection of regions that are MHC binding motif-dense increases the likelihood that the predicted polypeptide or peptide fragment contains a "valid" motif, and furthermore, that the reiteration of identical motifs may contribute to binding.

Additional MHC binding motif-based algorithms have been described by Parker *et al.*(1994) and Altuvia *et al.*(1994). In these algorithms, binding to a given MHC molecule is predicted by a linear function of the residues at each position, based on empirically defined parameters, and in the case of the Altuvia *et al.*(1994) algorithm, known crystallographic structures may also be taken into consideration.

Recombinant methods offer the opportunity to obtain well characterized epitopes of high purity for the production of diagnostic reagents and epitope-specific vaccine formulations (Mohapatra *et al.*, 1995). Based upon the amino acid sequence of a linear epitope and identification of the corresponding nucleotide sequence encoding same, polymerase chain reaction (PCR) may be performed to amplify the epitope-encoding region from cDNA. After cloning and expression in a suitable vector/host system, a large amount of epitopes of high purity can be extracted. Accordingly, the present invention clearly extends to both isolated non-recombinant polypeptides and recombinant polypeptides in an impure or isolated form.

The term "polypeptide" as used herein shall be taken to refer to any polymer consisting of amino acids linked by covalent bonds and includes within its scope the full-length amino acids disclosed herein, and any parts or fragments thereof such as,

for example, peptides consisting of about 5-50 amino acid residues in length, preferably about 5-30 amino acid residues in length, more preferably about 5-20 amino acid residues in length, and even more preferably about 5-10 amino acid residues in length. Also included within the scope of the definition of a "polypeptide" are amino acid sequence variants, containing one or more preferably conservative amino acid substitutions, deletions, or insertions, which do not alter at least one essential property of said polypeptide such as, for example, its immunogenicity, use as a diagnostic reagent, or effectiveness as a vaccine against *Lawsonia spp*, amongst others. Accordingly, a polypeptide may be isolated from a source in nature, or chemically synthesized. Furthermore, a polypeptide may be derived from a full-length protein by chemical or enzymatic cleavage, using reagents such as CNBr, trypsin, or chymotrypsin, amongst others.

Conservative amino acid substitutions are well-known in the art. For example, one or more amino acid residues of a native flagellar hook protein of the present invention can be substituted conservatively with an amino acid residue of similar charge, size or polarity, with the resulting polypeptide retaining an ability to function in a vaccine or as a diagnostic reagent as described herein. Rules for making such substitutions include those described by Dayhof (1978). More specifically, conservative amino acid substitutions are those that generally take place within a family of amino acids that are related in their side chains. Genetically-encoded amino acids are generally divided into four groups: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, and histidine; (3) non-polar=alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan; and (4) uncharged polar= glycine, asparagine, glutamine, cysteine, serine, threonine, and tyrosine. Phenylalanine, tyrosine and tryptophan are also jointly classified as aromatic amino acids. One or more replacements within any particular group such as, for example, the substitution of leucine for isoleucine or valine or alternatively, the substitution of aspartate for glutamate or threonine for serine, or of any other amino acid residue with a structurally-related amino acid residue, will generally have an insignificant effect on the function of the resulting polypeptide.

The present invention is not limited by the source of the subject immunogen and clearly extends to isolated and recombinant polypeptides which are derived from a natural or a non-natural occurring source.

The term "recombinant polypeptide" as used herein shall be taken to refer to a polypeptide which is produced *in vitro* or in a host cell by the expression of a genetic sequence encoding said polypeptide, which genetic sequence is under the control of a suitable promoter, wherein a genetic manipulation has been performed in order to achieve said expression. Accordingly, the term "recombinant polypeptide" clearly encompasses polypeptides produced by the expression of genetic sequences contained in viral vectors, cosmids or plasmids that have been introduced into prokaryotic or eukaryotic cells, tissues or organs. Genetic manipulations which may be used in this context will be known to those skilled in the art and include, but are not limited to, nucleic acid isolation, restriction endonuclease digestion, exonuclease digestion, end-filling using the Klenow fragment of *E. coli* DNA polymerase I or T4 DNA polymerase enzymes, blunt-ending of DNA molecules using T4 DNA polymerase or ExoIII enzymes, site-directed mutagenesis, ligation, and amplification reactions. As will be known to those skilled in the art, additional techniques such as nucleic acid hybridisations and nucleotide sequence analysis may also be utilised in the preparation of recombinant polypeptides, in confirming the identity of a nucleic acid molecule encoding a desired recombinant polypeptide and a genetic construct comprising the nucleic acid molecule.

Wherein the polypeptide of the present invention is a recombinant polypeptide, it may be produced in and, if desirable, isolated from a recombinant viral vector expression system or host cell. As will be known to those skilled in the relevant art, a cell for production of a recombinant polypeptide is selected on the basis of several parameters including the genetic constructs used to express the polypeptide under consideration, as well as the stability and activity of said polypeptide. It will also be known to those skilled in the art that the stability or activity of a recombinant

polypeptide may be determined, at least in part, by post-translational modifications to the polypeptide such as, for example, glycosylation, acylation or alkylation reactions, amongst others, which may vary between cell lines used to produce the recombinant polypeptide.

Accordingly, in a more particularly preferred embodiment, the present invention extends to a recombinant polypeptide or a derivative, homologue or analogue thereof as present in a virus particle, or as produced in prokaryotic or eukaryotic host cell, or in a virus or cell culture thereof.

The present invention also extends to a recombinant polypeptide according to any of the foregoing embodiments which is produced in a bacterial cell belonging to the genus *Lawsonia*, in particular a cell of *L. intracellularis,* or a culture thereof.

The term "isolated polypeptide" refers to a polypeptide of the present invention which has been purified to some extent, preferably to at least about 20% by weight of protein, preferably to at least about 50% by weight of protein, more preferably to at least about 60% by weight of protein, still more preferably to at least about 70% by weight of protein and even more preferably to at least about 80% by weight of protein or greater, from its natural source or, in the case of non-naturally-occurring polypeptides, from the culture medium or cellular environment in which it was produced. Such isolation may be performed to improve the immunogenicity of the polypeptide of the present invention, or to improve the specificity of the immune response against that polypeptide, or to remove toxic or undesirable contaminants therefrom. The necessary or required degree of purity of an isolated polypeptide will vary depending upon the purpose for which the polypeptide is intended, and for many applications it will be sufficient for the polypeptide preparation to contain no contaminants which would reduce the immunogenicity of the polypeptide when administered to a host animal, in particular a porcine or avian animal being immunized against PPE or, alternatively, which would inhibit immuno-specific binding in an immunoassay for the diagnosis of PPE or a causative agent thereof.

The purity of an isolated polypeptide of the present invention may be determined by any means known to those skilled in the art, including the degree of homogeneity of a protein preparation as assessed by SDS/polyacrylamide gel electrophoresis, 2-dimensional electrophoresis, or amino acid composition analysis or sequence analysis.

Preferably, the polypeptide of the present invention will be substantially homogeneous or substantially free of nonspecific proteins, as assessed by SDS/polyacrylamide gel electrophoresis, 2-dimensional electrophoresis, or amino acid composition analysis or sequence analysis.

The polypeptide of the present invention can be purified for use as a component of a vaccine composition by any one or a combination of methods known to those of ordinary skill in the art, including, for example, reverse phase chromatography, HPLC, ion-exchange chromatography, and affinity chromatography, among others.

In a preferred embodiment, the isolated or recombinant polypeptide of the invention functions is secretable into the periplasmic space of a cell, preferably into the periplasm of a prokaryotic cell, such as, for example, *Escherichia coli.* or *L. intracellularis,* or, alternatively, is immunologically cross-reactive with a *L. intracellularis* polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN.

In a particularly preferred embodiment, the isolated or recombinant polypeptide of the invention is derived from *Lawsonia spp.* or other pathogenic agent associated with the onset and/or development of PPE and more preferably, the subject polypeptide is derived from *L. intracellularis*.

A B-cell or T-cell epitope of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue,

analogue or derivative of any one or more of said polypeptides, may comprise one or more of the following:

(i) the primary amino acid sequence of any one of said polypeptides, as determined by an art-accepted methodology to comprise a continuous non-conformational epitope;

(ii) the secondary structure which any one of said polypeptides adopt, as determined by an art-accepted methodology to comprise a continuous conformational epitope;

(iii) the tertiary structure which any one of said polypeptides adopt in contact with another region of the same polypeptide molecule, as determined by an art-accepted methodology to comprise a discontinuous conformational epitope; or

(iv) the quaternary structure which any one of said polypeptides adopt in contact with a region of another polypeptide molecule, as determined by an art-accepted methodology to comprise a discontinuous conformational epitope.

Accordingly, immunogenic polypeptides or derivatives, homologues or analogues thereof comprising the same, or substantially the same primary amino acid sequence are hereinafter defined as "immunogens which comprise a B-cell or T-cell epitope" or similar term.

Immunogenic polypeptides or derivatives, homologues, or analogues thereof comprising different primary amino acid sequences may comprise immunologically identical immunogens, because they possess conformational B-cell or T-cell epitopes that are recognised by the immune system of a host species to be identical. Such immunogenic polypeptides or derivatives, homologues or analogues thereof are hereinafter defined as "immunogens which mimic or cross-react with a B-cell or T-cell epitope", or similar term.

Accordingly, the present invention extends to an immunogen which comprises, mimics, or cross-reacts with a B-cell or T-cell epitope of an isolated or recombinant polypeptide according to any one of the foregoing embodiments or a derivative, homologue or analogue thereof. In a particularly preferred embodiment, the present invention provides an immunogen which comprises, mimics, or cross-reacts with a B-cell or T-cell epitope of an isolated or recombinant polypeptide which in its native form is obtainable from a species of *Lawsonia* such as, but not limited to *L. intracellularis* and which polypeptide preferably has the same biological function as a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN, as hereinbefore defined.

Preferably, such immunogenic polypeptides will not comprise a primary amino acid sequence which is highly-conserved between *L. intracellularis* and another non-pathogenic microorganism which is normally resident in the gut or other organ of an animal, in particular a porcine or avian animal. The significance of this exclusion to those embodiments of the invention wherein specificity is essential to performance (eg vaccine and diagnostic applications) will be apparent to those skilled in the art.

To improve the immunogenicity of a subject polypeptide of the present invention one or more amino acids not corresponding to the original protein sequence can be added to the amino or carboxyl terminus of the polypeptide. Such extra amino acids are useful for coupling the polypeptide to another peptide or polypeptide, to a large carrier protein or to a solid support. Amino acids that are useful for these purposes include but are not limited to tyrosine, lysine, glutamic acid, aspartic acid, cysteine and derivatives thereof. Additional protein modification techniques can be used such as, e.g., NH_2-acetylation or COOH-terminal amidation, to provide additional means for coupling the polypeptide to another polypeptide or peptide molecule, or to a solid support. Procedures for coupling polypeptides to each other, or to carrier proteins or solid supports, are well known in the art. Polypeptides containing the above-mentioned extra amino acid residues at either the carboxyl- or amino-termini and either uncoupled or coupled to a carrier or solid support, are consequently within the

scope of the present invention.

Furthermore, the polypeptide can be immobilised to a polymeric carrier or support material.

In an alternative embodiment, the immunogenicity of a polypeptide of the present invention may be improved using molecular biology techniques to produce a fusion protein containing one or more polypeptides of the present invention fused to a carrier molecules such as a highly immunogenic protein. For example, a fusion protein containing a polypeptide of the present invention fused to the highly immunogenic B subunit of cholera toxin can be used to increase the immune response to the polypeptide. The present invention also contemplates fusion proteins comprising a cytokine, such as an interleukin, fused to the subject polypeptide of the present invention, and genes encoding same.

Preferably, the polypeptide of the present invention, or a derivative, homologue or analogue thereof, when administered to a mammal, induces an immune response in said mammal. More preferably, the polypeptide of the present invention, when administered to a mammal, in particular a porcine animal (e.g., a pig) induces a protective immune response against *Lawsonia spp.*, and preferably against *L. intracellularis,* therein. As used herein, the phrase "induction of a protective immune response", and the like, refers to the ability of the administered polypeptide of the present invention to prevent or detectably slow the onset, development, or progression of symptoms associated with *Lawsonia* infection, and preferably, to prevent or detectably slow the onset, development, or progression of symptoms associated with PPE in pigs.

Preferably, the isolated or recombinant immunogenic polypeptide is selected from the group consisting of the following:

(i) a polypeptide which comprises an amino acid sequence which has at least about 60% sequence identity overall to an amino acid sequence selected

from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18;

(ii) a polypeptide which comprises an amino acid sequence which has at least about 60% sequence identity overall to an amino acid sequence encoded by *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a polypeptide which comprises at least about 5 contiguous amino acids, of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18;

(iv) a polypeptide which comprises at least about 5 contiguous amino acids of an amino acid sequence encoded by *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(v) a homologue, analogue or derivative of any one of (i) to (iv) which mimics a B-cell or T-cell epitope of *Lawsonia spp.*

In an alternative preferred embodiment, the isolated or recombinant immunogenic polypeptide is selected from the group consisting of the following:

(i) a polypeptide which comprises an amino acid sequence encoded by a nucleotide sequence having at least about 60% sequence identity overall to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(ii) a polypeptide which comprises an amino acid sequence encoded by a nucleotide sequence having at least about 60% sequence identity overall to

the nucleotide sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a polypeptide encoded by at least about 15 contiguous nucleotides of a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(iv) a polypeptide encoded by at least about 15 contiguous nucleotides of a nucleotide sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(v) a homologue, analogue or derivative of any one of (i) to (iv) which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

Preferably, the immunogenic polypeptide encompassed by the present invention has at least about 70% identity, more preferably at least about 80% identity, even more preferably at least about 90% identity, and still even more preferably at least about 95% identity to the amino acid sequence of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, as hereinbefore defined.

In determining whether or not two amino acid sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences will arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context,

reference to a percentage sequence identity or similarity between two or more amino acid sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. For example, amino acid sequence identities or similarities may be calculated using the GAP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984). The GAP programme utilizes the algorithm of Needleman and Wunsch (1970) to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment. Alternatively or in addition, where more than two amino acid sequences are being compared, the ClustalW programme of Thompson *et al* (1994) can be used.

Preferably, the isolated or recombinant immunogenic polypeptide of the invention comprises at least about 10 contiguous amino acids of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, as hereinbefore defined. More preferably, the isolated or recombinant immunogenic polypeptide of the invention comprises at least about 20 contiguous amino acid residues of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, as hereinbefore defined. Even more preferably, the isolated or recombinant immunogenic polypeptide of the invention comprises at least about 30 contiguous amino acid residues of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, as hereinbefore defined, and still even more preferably, at least about 40 contiguous amino acid residues of said flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, or ytfN polypeptides.

The present invention further encompasses homologues, analogues and derivatives of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, as hereinbefore defined.

"Homologues" of a polypeptide are those immunogenic polypeptides that are derived

from a full-length *L. intracellularis* polypeptides described herein, or have sequence similarity to a full-length *L. intracellularis* polypeptide, notwithstanding one or more amino acid substitutions, deletions and/or additions relative to the full-length *L. intracellularis* polypeptide. A homologue may also retain the biological activity or catalytic activity of the full-length polypeptide. In such homologues, one or more amino acids can be replaced by other amino acids having similar properties such as, for example, hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α-helical structures of β-sheet structures, and so on.

Substitutional variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein. Insertions can comprise amino-terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the sequence.

Amino acid variants of the polypeptide of the present invention may readily be made using polypeptide synthetic techniques well known in the art, such as solid phase synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional,

insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

"Analogues" are defined as those immunogenic polypeptides that are derived from a full-length *L. intracellularis* polypeptides described herein, or have sequence similarity to a full-length *L. intracellularis* polypeptide, notwithstanding one or more non-naturally occurring or modified amino acid residues relative to the naturally-occurring full-length *L. intracellularis* polypeptide. The term "analogue" shall also be taken to include an amino acid sequence which is not similar to an amino acid sequence of a full-length *L. intracellularis* polypeptide set forth herein, however mimics or cross-reacts with a B-cell or T-cell epitope of *Lawsonia spp.* and preferably, mimics or cross-reacts with a B-cell or T-cell epitope of *L. intracellularis,* such as, for example, a polypeptide which is derived from a computational prediction or empirical data revealing the secondary, tertiary or quaternary structure of the full-length polypeptide or an epitope thereof.

For example, mimotopes (polypeptide analogues that cross-react with a B-cell or T-cell epitope of the *Lawsonia* polypeptide of the invention but, however, comprise a different amino acid sequence to said epitope) may be identified by screening random amino acid sequences in polypeptide libraries with antibodies that bind to a desired T-cell or B-cell epitope. As with techniques for the identification of B-cell or T-cell epitopes as described *supra*, the antibodies used to identify such mimotopes may be polyclonal or monoclonal or recombinant antibodies, in crude or purified form. Mimotopes of a T-cell epitope may then be assayed further for their ability to stimulate T-cell cytotoxic or proliferative responses *in vitro.* Mimotopes are particularly useful as analogues of non-linear (i.e., conformational) epitopes of the polypeptide of the present invention, because conformational epitopes are generally formed from non-contiguous regions in a polypeptide, and the mimotopes provide immunogenic equivalents thereof in the form of a single polypeptide molecule.

Additionally, the use of polypeptide analogues can result in polypeptides with increased immunogenic and/or antigenic activity, that are less sensitive to enzymatic degradation, and which are more selective. A suitable proline analogue is 2-aminocyclopentane carboxylic acid (βAC^5c) which has been shown to increase the immunogenic activity of a native polypeptide more than 20 times (Mierke *et al*, 1990; Portoghese *et al*, 1990; Goodman *et al*, 1987).

"Derivatives" of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, as hereinbefore defined, are those peptides or polypeptides which comprise at least about five contiguous amino acid residues of any one or more of said flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, or ytfN polypeptides.

A "derivative" may further comprise additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a flhB, or fliR, or ntrC, or glnH, or motA, or motB, or tlyC, or ytfM, or ytfN polypeptide, as hereinbefore defined. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents such as, for example, a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence of a flhB, or fliR, or ntrC, or glnH, or motA, or motB, or tlyC, or ytfM, or ytfN polypeptide, such as, for example, a reporter molecule which is bound thereto to facilitate its detection.

Other examples of recombinant or synthetic mutants and derivatives of a polypeptide immunogen of the present invention include those incorporating single or multiple substitutions in the amino acid sequence of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides. Recombinant or synthetic mutants and derivatives produced by making deletions from the amino acid sequence of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, are also

included within the scope of preferred derivatives. Additionally, recombinant or synthetic mutants and derivatives produced by making additions to a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, such as, for example, using carbohydrates, lipids and/or proteins or polypeptides, are also encompassed by the present invention.

Naturally-occurring or altered glycosylated or acylated forms of the flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, or ytfN polypeptides are particularly contemplated by the present invention.

Additionally, homopolymers or heteropolymers comprising one or more copies of the reference polypeptides, or one or more derivatives, homologues or analogues thereof, are clearly within the scope of the present invention.

Preferably, homologues, analogues and derivatives of the flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, or ytfN polypeptides of the invention are "immunogenic", defined hereinafter as the ability of said polypeptide, or a derivative, homologue or analogue thereof, to elicit B cell and/or T cell responses in the host, in response to immunization.

Preferred homologues, analogues and derivatives of the flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, or ytfN polypeptides of the invention include any amino acid variant that functions as B cell or T cell epitope of any one of said polypeptides, wherein said variant is capable of mediating an immune response, such as, for example, a mimotope of the immunogenic polypeptide which has been produced by synthetic means, such as by Fmoc chemistry. The only requirement of such variant molecules is that they cross-react immunologically with a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN, as hereinbefore defined, or an epitope of said polypeptide.

As will be apparent to those skilled in the art, such homologues, analogues and

derivatives of the polypeptides of the invention molecules will be useful to prepare antibodies that cross-react with antibodies against said polypeptide and/or to elicit a protective immune response of similar specificity to that elicited by said polypeptide. Such molecules will also be useful in diagnostic and other applications that are immunological in nature such as, for example, diagnostics which utilise one or more immunoassay formats (eg. ELISA, RIA and the like).

Accordingly, the immunogen of the present invention or a derivative, homologue or analogue thereof is useful in vaccine compositions that protect an individual against infection by *L. intracellularis* and/or as an antigen to elicit polyclonal or monoclonal antibody production and/or in the detection of antibodies against *L. intracellularis* in infected animals, particularly in porcine and avian animals.

The polypeptides of the present invention may comprise leader sequences to facilitate their secretion into the periplasmic space, either as part of the native protein, or alternatively, added by recombinant engineering means. Such may have improved immunogenicity compared to non-secreted or non-secretable polypeptides of *L. intracellularis*, or non-secreted or non-secretable polypeptides of other causative agents of PPE. The particular advantages of such peptides will be immediately apparent to those skilled in the production of vaccine compositions, where the inherent immunogenicity of the immunogen is an important consideration for a protective immune response to be conferred.

Moreover, unique regions of the *L. intracellularis* polypeptides exemplified herein are promising antigenic peptides for the formulation of *Lawsonia*-specific vaccines and diagnostics for the specific detection of *Lawsonia spp.* in biological samples.

A second aspect of the present invention provides a vaccine composition for the prophylaxis or treatment of infection in a mammal or bird by *L. intracellularis* or similar or otherwise related microorganism, said vaccine composition comprising:

(i) an immunogenic component which comprises an isolated or

recombinant polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides or an immunogenic homologue, analogue or derivative of any one of said polypeptides which is immunologically cross-reactive with *L. intracellularis;* and

(ii) one or more carriers, diluents and/or adjuvants suitable for veterinary or pharmaceutical use.

As used herein, the term "immunogenic component" refers to a polypeptide encoded by DNA from, or derived from, *L. intracellularis* or a related microorganism thereto which is capable of inducing a protective immune response in an animal, in particular a porcine or avian animal, whether or not said polypeptide is in an isolated or recombinant form. Accordingly, the vaccine composition clearly encompasses those vaccine compositions which comprise attenuated, killed or non-pathogenic isolates or forms of *L. intracellularis* or related microorganisms thereto which comprise or express said polypeptide.

By "protective immune response" is meant that the immunogenic component elicits an immune response in the animal to which the vaccine composition is administered at the humoral and/or cellular level which is sufficient to prevent infection by *L. intracellularis* or a related microorganism thereto and/or which is sufficient to detectably reduce one or more symptoms or conditions, or to detectably slow the onset of one or more symptoms or conditions, associated with infection by *L. intracellularis* or a related microorganism thereto in an animal host, as compared to a control infected animal. The term "effective amount" of an immunogenic component present in the vaccine composition refers to that amount of said immunogenic component that is capable of inducing a protective immune response after a single complete dose has been administered, or after several divided doses have been administered.

Preferably, the polypeptide component of the subject vaccine composition comprises an amino acid sequence which is both immunogenic and specific, by virtue of its

immunological cross-reactivity with the causative agent of PPE, *L. intracellularis*. In this regard, it will be apparent from the preceding description that such polypeptide components may comprise the amino acid sequence of a polypeptide of *L. intracellularis* as exemplified herein, or alternatively, an immunologically cross-reactive homologue, analogue or derivative of said amino acid sequence, such as, for example, a mimotope of said sequence.

The immunogenic polypeptide or immunogenic homologue, analogue or derivative may be a naturally-occurring polypeptide in isolated or recombinant form according to any of the embodiments described *supra* or exemplified herein. Preferably, the immunogenic polypeptide or immunogenic homologue, analogue or derivative is derived from *Lawsonia spp.*, in particular *L. intracellularis* or a microorganism that is related thereto.

Preferably, the immunogenic component has undergone at least one purification step or at least partial concentration from a cell culture comprising *L. intracellularis* or a related microorganism thereto, or from a lysed preparation of *L. intracellularis* cells or related microorganism, or from another culture in which the immunogenic component is recombinantly expressed. The purity of such a component which has the requisite immunogenic properties is preferably at least about 20% by weight of protein in a particular preparation, more preferably at least about 50%, even more preferably at least about 60%, still more preferably at least about 70% and even more preferably at least about 80% or greater.

The immunogenic component of the vaccine of the present invention can comprise a single polypeptide, or a range or combination of different polypeptides covering different or similar epitopes. In addition or, alternatively, a single polypeptide can be provided with multiple epitopes. The latter type of vaccine is referred to as a polyvalent vaccine. A multiple epitope includes two or more epitopes located within a polypeptide molecule.

The formulation of vaccines is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pennsylvania, USA.

A particularly useful form of the vaccine is a recombinant vaccine produced, for example, in a vaccine vector, such as but not limited to a mammalian cell transfected with a vaccinia virus vector, an insect cell transfected with a baculovirus vector, or a bacterial cell transfected with a plasmid or cosmid, the only requirement being that the vector expresses the immunogenic component.

The present invention clearly extends to recombinant vaccine compositions in which the immunogenic component at least is contained within killed vaccine vectors prepared, for example, by heat, formalin or other chemical treatment, electric shock or high or low pressure forces. According to this embodiment, the immunogenic component of the vaccine is generally synthesized in a live vaccine vector which is killed prior to administration to an animal.

Furthermore, the vaccine vector expressing the immunogenic component may be non-pathogenic or attenuated. Within the scope of this embodiment are cells that have been transfected with non-pathogenic or attenuated viruses encoding the immunogenic component of the vaccine and non-pathogenic or attenuated cells that directly express the immunogenic component.

Attenuated or non-pathogenic host cells include those cells which are not harmful to an animal to which the subject vaccine is administered. As will be known to those skilled in the art, "live vaccines" can comprise an attenuated virus vector encoding the immunogenic component or a host cell comprising same, which is capable of replicating in an animal to which it is administered, and using host cell machinery to express the immunogenic component albeit producing no adverse side-effects therein. Such vaccine vectors may colonise the gut or other organ of the vaccinated animal. Such live vaccine vectors are efficacious by virtue of their ability to continually

express the immunogenic component in the host animal for a time and at a level sufficient to confer protective immunity against a pathogen which expresses an immunogenic equivalent of said immunogenic component. The present invention clearly encompasses the use of such attenuated or non-pathogenic vectors and live vaccine preparations.

The vaccine vector may be a virus, bacterial cell or a eukaryotic cell such as an insect, avian, porcine or other mammalian cell or a yeast cell or a cell line such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDCK cell lines. Suitable prokaryotic cells include *Mycobacterium spp., Corynebacterium spp., Salmonella spp., Escherichia coli, Bacillus spp.* and *Pseudomonas spp*, amongst others. Bacterial strains which are suitable for the present purpose are well-known in the relevant art (Ausubel *et al*, 1987; Sambrook *et* al, 1989).

Such cells and cell lines are capable of expression of a genetic sequence encoding a polypeptide of the present invention from *L. intracellularis*, or a homologue, analogue or derivative thereof, in a manner effective to induce a protective immune response in the animal. For example, a non-pathogenic bacterium can be prepared containing an expression vector which comprises a nucleotide sequence encoding a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue, analogue, or derivative thereof, wherein said nucleotide sequence is placed operably under the control of a constitutive or inducible promoter sequence. The bacterium is then permitted to colonise suitable locations in a pig's gut, where it replicates and expresses the said polypeptide in amount sufficient to induce a protective immune response against *L. intracellularis*.

In a further alternative embodiment, the vaccine can be a DNA or RNA vaccine comprising a DNA or RNA molecule encoding a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides or

homologues, analogues or derivatives thereof, wherein said vaccine is injected into muscular tissue or other suitable tissue in a pig under conditions sufficient to permit transient expression of said DNA or RNA to produce an effective amount of said polypeptide to induce a protective immune response. In a preferred embodiment, the DNA vaccine is in the form of a plasmid, in which the DNA is operably connected with a promoter region capable of expressing the nucleotide sequence encoding the immunogen in cells of the immunized animal.

In the production of a recombinant vaccine, except for a DNA vaccine described herein, it is therefore necessary to express the immunogenic component in a suitable vector system. For the present purpose, the immunogenic component can be expressed by:

(i) placing an isolated nucleic acid molecule in an expressible format, said nucleic acid molecule comprising the coding region of a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes, or a protein-encoding homologue, analogue or derivative thereof;

(ii) introducing the isolated nucleic acid molecule of (i) in an expressible format into a suitable vaccine vector; and

(iii) incubating or growing the vaccine vector for a time and under conditions sufficient for expression of the immunogenic component encoded by said nucleic acid molecule to occur.

It will be apparent from the preceding discussion that the protein-encoding region of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, and 17, or alternatively or in addition, a protein-encoding nucleotide sequence of *L. intracellularis* DNA contained within a deposited plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and

NM01/23286 (plasmid pGTE#8 ytfN).

Preferred homologues of the protein-encoding region of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene include those nucleotide sequences selected from the group consisting of:

(i) a protein-encoding nucleotide sequence having at least about 60% sequence identity overall to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17 or a degenerate variant thereof;

(ii) a protein-encoding nucleotide sequence having at least about 60% sequence identity overall to the protein-encoding sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a protein-encoding nucleotide sequence which comprises at least about 15 contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(iv) a protein-encoding nucleotide sequence which comprises at least about 15 contiguous nucleotides of the protein-encoding sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(v) a protein-encoding nucleotide sequence which hybridizes under at least low stringency conditions to the complement of a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15,

and 17; and

(vi) a protein-encoding nucleotide sequence which hybridizes under at least low stringency conditions to the non-coding strand of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN).

The present invention clearly extends to analogues or derivatives of any one of (i) to (vi) which encode a polypeptide which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

For the present purpose, a preferred homologue of the protein-encoding region of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene will have at least about 80% nucleotide sequence identity to the coding region of said gene, still more preferably at least about 90% identity, and yet still more preferably at least about 95% identity.

In determining whether or not two nucleotide sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences may arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity between two or more nucleotide sequences shall be taken to refer to the number of identical residues between said sequences as determined using any standard algorithm known to those skilled in the art. For example, nucleotide sequences may be aligned and their identity calculated using the BESTFIT programme or other appropriate programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America

(Devereaux *et al*, 1984).

Preferably, a homologue of the protein-encoding region of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene hybridizes under at least medium stringency conditions to the non-coding strand of said gene, even more preferably under high stringency conditions to the non-coding strand of said gene.

For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28°C. A moderate stringency is defined herein as being a hybridisation and/or washing carried out in 2xSSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C. A high stringency is defined herein as being a hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS, or lower salt concentration, and at a temperature of at least 65°C. Reference herein to a particular level of stringency encompasses equivalent conditions using wash/hybridization solutions other than SSC known to those skilled in the art.

Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Those skilled in the art will be aware that the conditions for hybridisation and/or wash may vary depending upon the nature of the hybridisation membrane or the type of hybridisation probe used. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification of the parameters affecting hybridisation between nucleic acid molecules, reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

As used herein, a "nucleic acid molecule in an expressible format" is a protein-encoding region of a nucleic acid molecule placed in operable connection with a promoter or other regulatory sequence capable of regulating expression in the vaccine vector system.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e., upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. In the present context, the term "promoter" is also used to describe a recombinant, synthetic or fusion molecule, or derivative which confers, activates or enhances the expression of a nucleic acid molecule to which it is operably connected, and which encodes the immunogenic polypeptide. Preferred promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or to alter the spatial expression and/or temporal expression of the said nucleic acid molecule.

Placing a nucleic acid molecule under the regulatory control of, i.e., "in operable connection with", a promoter sequence means positioning said molecule such that expression is controlled by the promoter sequence. Promoters are generally, but not necessarily, positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

The prerequisite for producing intact polypeptides in bacteria such as *E. coli* is the

use of a strong promoter with an effective ribosome binding site. Typical promoters suitable for expression in bacterial cells such as *E. coli* include, but are not limited to, the *lacz* promoter, temperature-sensitive λ_L or λ_R promoters, T7 promoter or the IPTG-inducible *tac* promoter. A number of other vector systems for expressing the nucleic acid molecule of the invention in *E. coli* are well-known in the art and are described, for example, in Ausubel *et al* (1987) or Sambrook *et al* (1989). Numerous plasmids with suitable promoter sequences for expression in bacteria and efficient ribosome binding sites have been described, such as for example, pKC30 (λ_L: Shimatake and Rosenberg, 1981); pKK173-3 (*tac*: Amann and Brosius, 1985), pET-3 (T7: Studier and Moffat, 1986); the pBAD/TOPO or pBAD/Thio-TOPO series of vectors containing an arabinose-inducible promoter (Invitrogen, Carlsbad, CA), the latter of which is designed to also produce fusion proteins with thioredoxin to enhance solubility of the expressed protein; the pFLEX series of expression vectors (Pfizer Inc., CT, USA); or the pQE series of expression vectors (Qiagen, CA), amongst others. Typical promoters suitable for expression in viruses of eukaryotic cells and eukaryotic cells include the SV40 late promoter, SV40 early promoter and cytomegalovirus (CMV) promoter, CMV IE (cytomegalovirus immediate early) promoter amongst others.

Means for introducing the isolated nucleic acid molecule or a genetic construct comprising same into a cell for expression of the immunogenic component of the vaccine composition are well-known to those skilled in the art. The technique used for a given organism depends on the known successful techniques. Means for introducing recombinant DNA into animal cells include microinjection, transfection mediated by DEAE-dextran, transfection mediated by liposomes such as by using lipofectamine (Gibco, MD, USA) and/or cellfectin (Gibco, MD, USA), PEG-mediated DNA uptake, electroporation and microparticle bombardment such as by using DNA-coated tungsten or gold particles (Agracetus Inc., WI, USA) amongst others.

The immunogenic component of a vaccine composition as contemplated herein exhibits excellent therapeutic activity, for example, in the treatment and/or

prophylaxis of PPE when administered in an amount which depends on the particular case. For example, for recombinant polypeptide molecules, from about 0.5 μg to about 20 mg may be administered, preferably from about 1 μg to about 10 mg, more preferably from about 10 μg to about 5 mg, and most preferably from about 50 μg to about 1 mg equivalent of the immunogenic component in a volume of about 1ml to about 5ml. For DNA vaccines, a preferred amount is from about 0.1 μg/ml to about 5 mg/ml in a volume of about 1 to about 5 ml. The DNA can be present in "naked" form or it can be administered together with an agent facilitating cellular uptake (e.g., in liposomes or cationic lipids). The important feature is to administer sufficient immunogen to induce a protective immune response. The above amounts can be administered as stated or calculated per kilogram of body weight. Dosage regime can be adjusted to provide the optimum therapeutic response. For example, several divided doses can be administered or the dose can be proportionally reduced as indicated by the exigencies of the therapeutic situation. Booster administration may also be required.

The vaccine of the present invention can further comprise one or more additional immunomodulatory components such as, for example, an adjuvant or cytokine molecule, amongst others, that is capable of increasing the immune response against the immunogenic component. Non-limiting examples of adjuvants that can be used in the vaccine of the present invention include the RIBI adjuvant system (Ribi Inc., Hamilton, MT, USA), alum, mineral gels such as aluminium hydroxide gel, oil-in-water emulsions, water-in-oil emulsions such as, for example, Block co-polymer (CytRx, Atlanta GA, USA),QS-21 (Cambridge Biotech Inc., Cambridge MA, USA), SAF-M (Chiron, Emeryville CA, USA), AMPHIGEN® adjuvant, Freund's complete adjuvant; Freund's incomplete adjuvant; and Saponin, QuilA or other saponin fraction, monophosphoryl lipid A, and Avridine lipid-amine adjuvant. Other immunomodulatory agents that can be included in the vaccine include, for example, one or more cytokines, such as interferon and/or interleukin, or other known cytokines. Non-ionic surfactants such as, for example, polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether may also be included in the vaccines of the present invention.

The vaccine composition can be administered in a convenient manner such as by oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or by implantation (eg., using slow release technology), provided that a sufficient degree of the immunogenicity of the immunizing antigen is retained for the purposes of eliciting an immune response in the animal being treated. Depending on the route of administration, the immunogenic component may be required to be coated in a material to protect it from the action of enzymes, acids and other natural conditions which may inactivate it, such as those in the digestive tract.

The vaccine composition may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, or in oils. Under ordinary conditions of storage and use, these preparations can contain a preservative to prevent the growth of microorganisms. Alternatively, the vaccine composition can be stored in lyophilised form to be rehydrated with an appropriate vehicle or carrier prior to use.

Pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be fluid to the extent that easy syringability exists, unless the pharmaceutical form is a solid or semi-solid such as when slow release technology is employed. In any event, it must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms.

The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents such as, for example,, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents such as, for example,, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption such as, for example,, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter-sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients selected from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

The present invention extends to vaccine compositions which confer protection against infection by one or more isolates or sub-types of *L. intracellularis* including those that belong to the same serovar or serogroup as *L. intracellularis*. The vaccine composition preferably also confers protection against infection by other species of the genus *Lawsonia* or other microorganisms related thereto, as determined at the nucleotide, biochemical, structural, physiological and/or immunointeractive level; the only requirement being that said other species or other microorganism expresses a polypeptide which is immunologically cross-reactive to a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue, analogue or derivative of any one or more of said polypeptides as described herein. For example, such related microorganisms may comprise genomic DNA which is at least about 70% identical overall to the genomic

DNA of *L. intracellularis* as determined using standard genomic DNA hybridisation and analysis techniques.

The terms "serogroup" and "serovar" relate to a classification of microorganisms which is based upon serological typing data, in particular data obtained using agglutination assays such as the microscopic agglutination test (MAT). Those skilled in the art will be aware that serovar and serogroup antigens are a mosaic on the cell surface and, as a consequence there will be no strict delineation between bacteria belonging to a serovar and/or serogroup. Moreover, organisms which belong to different species may be classified into the same serovar or serogroup because they are indistinguishable by antigenic determination. As used herein, the term "serovar" means one or more *Lawsonia* strains which are antigenically-identical with respect to antigenic determinants produced by one or more loci. Quantitatively, serovars may be differentiated from one another by cross-agglutination absorption techniques. As used herein, the term "serogroup" refers to a group of *Lawsonia spp.* whose members cross-agglutinate with shared group antigens and do not cross-agglutinate with the members of other groups and, as a consequence, the members of a serogroup have more or less close antigenic relations with one another by simple cross-agglutination.

The present invention thus clearly extends to vaccine compositions for the treatment and/or prophylaxis of animals, in particular, vaccine compositions for the treatment and/or prophylaxis of porcine and/or avian species, against any bacterium belonging to the same serovar or serogroup as *L. intracellularis.* Preferably, such organisms will express a polypeptide homologue, analogue or derivative of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides.

The present invention extends further to vaccine compositions capable of conferring protection against a "genetic variant" of *L. intracellularis*, the only requirement being that said variant expresses a polypeptide which is immunologically cross-reactive to

a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides. Genetic variants of *L. intracellularis* can be developed by mutation, recombination, conjugation or transformation of *L. intracellularis* or may occur naturally. It will be known to a person skilled in the art how to produce such derivatives.

In a particularly preferred embodiment, the vaccine composition of the invention is intended for or suitable for the prophylaxis and/or treatment of infection in a porcine or avian animal and more preferably, for prophylaxis and/or treatment of a porcine animal for infection by *L. intracellularis.*

Accordingly, the present invention clearly extends to the use of the immunogenic polypeptide of the invention or a DNA or RNA molecule encoding the same, according to any one of the preceding embodiments or as exemplified herein in the preparation of a medicament for the treatment and/or prophylaxis of PPE in animals, particularly porcine or avian animals.

The invention further extends to a method of treatment and/or prophylaxis of PPE in an animal such as an avian or porcine animal, said method comprising administering the vaccine composition or the immunogenic polypeptide of the invention or a DNA or RNA molecule encoding the same, as described or exemplified herein to said animal for a time and under conditions sufficient for an immune response to occur thereto. Preferably, in the case of administration of a vaccine composition, the immune response to the immunogen is a protective immune response.

Those skilled in the art will recognise the general applicability of the invention in vaccinating animals other than porcine and avian animals against *L. intracellularis* and/or related microorganisms. In the general application of the vaccine of the present invention, the only prerequisite is that the animal on which protection is conferred is capable of being infected with *L. intracellularis* and/or a related microorganism thereto and that, in the case of a related microorganism to *L.*

intracellularis, said related microorganism expresses a B-cell or T-cell epitope which mimics or cross-reacts with the polypeptide component of the vaccine composition described herein. Animals which may be protected by the vaccine of the present invention include, but are not limited to, humans, primates, companion animals (e.g., cats, dogs), livestock animals (e.g., pigs, sheep, cattle, horses, donkeys, goats), laboratory test animals (e.g., mice, rats, guinea pigs, rabbits) and captive wild animals (e.g., kangaroos, foxes, deer). The present invention also extends to the vaccination of birds such as poultry birds, game birds and caged birds.

The present invention further extends to combination vaccines comprising an effective amount of a first immunogenic component comprising a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue, analogue or derivative thereof as described herein, or a DNA or RNA molecule encoding the same, combined with an effective amount of a second immunogenic component comprising one or more other antigens capable of protecting a porcine animal, or bird, against either *Lawsonia spp.* or another pathogen that infects and causes disease in said animal. The second immunogenic component is different from the first immunogenic component and is preferably selected from the group consisting of the *L. intracellularis* FlgE, hemolysin, OmpH, SodC, flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides and homologues, analogues or derivatives thereof. The present invention clearly extends to DNA vaccines and vaccine vectors which express said first immunogenic component and said second immunogenic component.

It is within the scope of the invention to encompass vaccine compositions comprising multimeric and polymeric forms of any one or more of the immunogenic polypeptides described herein, such as tandem arrays of homologous amino acid sequences, or, alternatively, tandem arrays of heterologous immunogenic repeats of amino acid sequences. The present invention extends further to nucleic acid molecules encoding such polymeric forms.

The isolated or recombinant polypeptide of the invention, or an immunologically-equivalent homologue, analogue or derivative thereof is also useful for the preparation of immunologically interactive molecules which are useful in the diagnosis of infection of an animal by *Lawsonia spp.*, in particular by *L. intracellularis* or a related organism thereto.

As used herein, the term "immunologically interactive molecule" includes antibodies and antibody derivatives and functional equivalents, such as a Fab, or a SCAB (single-chain antibody), any of which optionally can be conjugated to an enzyme, radioactive or fluorescent tag, amongst others. The only requirement of such immunologically interactive molecules is that they are capable of binding specifically to the immunogenic polypeptide of the present invention as hereinbefore described.

Accordingly, a further aspect of the invention extends to an immunologically interactive molecule which is capable of binding to a polypeptide selected from the group consisting of:

(i) a polypeptide which comprises an amino acid sequence which has at least about 60% sequence identity overall to an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18;

(ii) a polypeptide which comprises an amino acid sequence which has at least about 60% sequence identity overall to an amino acid sequence encoded by *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a polypeptide which comprises at least about 5 contiguous amino acids of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, and 18;

(iv) a polypeptide which comprises at least about 5 contiguous amino acids

of an amino acid sequence encoded by *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(v) a polypeptide which comprises an amino acid sequence encoded by a nucleotide sequence having at least about 60% sequence identity overall to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(vi) a polypeptide which comprises an amino acid sequence encoded by a nucleotide sequence having at least about 60% sequence identity overall to the nucleotide sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(vii) a polypeptide encoded by at least about 15 contiguous nucleotides of a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(viii) a polypeptide encoded by at least about 15 contiguous nucleotides of a nucleotide sequence of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN); and

(ix) a homologue, analogue or derivative of any one of (i) to (viii) which mimics a B-cell or T-cell epitope of *Lawsonia spp*.

In a preferred embodiment, the immunologically interactive molecule is an antibody that binds specifically to one or more epitopes of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides. More preferably, the immunologically interactive molecule binds specifically to one or more epitopes of a polypeptide from a causative agent of PPE, such as, for example, *L. intracellularis*.

Conventional methods can be used to prepare the immunologically interactive molecules. For example, by using a polypeptide immunogen of the present invention, polyclonal antisera or monoclonal antibodies can be made using standard methods. For example, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the polypeptide of the present invention which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a polypeptide include conjugation to carriers, or other techniques well known in the art. For example, the polypeptide can be administered in the presence of adjuvant or can be coupled to a carrier molecule, as known in the art, that enhances the immunogenicity of the polypeptide. The progress of immunization can be monitored by detection of antibody titres in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, for example, IgG molecules corresponding to the polyclonal antibodies can be isolated from the antisera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an animal immunised with a polypeptide of the present invention and fused with myeloma cells by standard somatic cell fusion procedures, thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, for example, the hybridoma technique originally developed by Kohler and Milstein (1975), as well as other techniques such as the human B-cell hybridoma technique (Kozbor *et al.*, 1983), the EBV-hybridoma technique to produce

human monoclonal antibodies (Cole *et al.*, 1985), and screening of combinatorial antibody libraries (Huse *et al.*, 1989). Hybridoma cells can be isolated and screened immunochemically for production of antibodies that are specifically reactive with the polypeptide and monoclonal antibodies isolated therefrom.

As with all immunogenic compositions for eliciting antibodies, the immunogenically effective amounts of the peptides of the invention must be determined empirically. Factors to be considered include the immunogenicity of the native polypeptide, whether or not the polypeptide will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier, the route of administration for the composition, i.e., intravenous, intramuscular, subcutaneous, *etc.*, and the number of immunizing doses to be administered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The term "antibody" as used herein, is intended to include fragments thereof which are also specifically reactive with a polypeptide that mimics or cross-reacts with a B-cell or T-cell epitope of the *L. intracellularis* polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

It is within the scope of this invention to include any secondary antibodies (monoclonal, polyclonal or fragments of antibodies), including anti-idiotypic antibodies, directed to the first mentioned antibodies discussed above. Both the first and second antibodies can be used in detection assays or a first antibody can be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of a polypeptide

which mimics, or cross-reacts with a B-cell or T-cell epitope of a *L. intracellularis* polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides.

The antibodies described herein are useful for determining B-cell or T-cell epitopes of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, such as, for example, by testing the ability of synthetic peptides to cross-react immunologically with said polypeptide or to elicit the production of antibodies which cross-react with said polypeptide. Using methods described herein, polyclonal antibodies, monoclonal antibodies or chimeric monoclonal antibodies can also be raised to peptides which mimic or cross-react with a B-cell or T-cell epitope of a *L. intracellularis* polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides.

More particularly, the polyclonal, monoclonal or chimeric monoclonal antibodies can be used to detect the polypeptide of the invention and/or any homologues, analogues or derivatives thereof, in various biological materials. For example, they can be used in an ELISA, radioimmunoassay, or histochemical test. In other words, the antibodies can be used to test for binding to a polypeptide of the invention or to a homologue, analogue or derivative thereof, in a biological sample to diagnose the presence of *L. intracellularis* therein.

Accordingly, a further aspect of the invention provides a method of diagnosing infection of an animal by *L. intracellularis* or a related microorganism thereto, said method comprising the steps of contacting a biological sample derived from said animal with an immunologically interactive molecule which is capable of binding to a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or a homologue, analogue or derivative thereof, for a time and under conditions sufficient for an antigen:antibody complex to form, and detecting said complex formation.

According to this embodiment of the present invention, the immunologically interactive molecule is preferably an antibody molecule prepared against a *L. intracellularis* polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or an analogue or derivative thereof.

If the biological sample being tested contains one or more epitopes of a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, or an immunologically cross-reactive homologue, analogue or derivative thereof, it will give a positive binding result to the immunologically interactive molecule.

Preferably, the biological sample is derived from a porcine or avian host of the pathogen *L. intracellularis* or a related microorganism thereto, and includes an appropriate tissue or fluid sample from the animal.

Preferred biological samples are derived from the ileum, caecum, small intestine, large intestine, whole serum or lymph nodes of the porcine or avian host animal being tested. Alternatively or in addition the biological test sample may comprise faeces or a rectal swab derived from the animal.

To distinguish *L. intracellularis* from other microorganisms resident in the gut or other organ of an animal, the antibodies should not be prepared against highly-conserved epitopes of the *L. intracellularis* polypeptide, such as, for example, those amino acid sequences of at least 5 amino acids in length which are conserved between *L. intracellularis* and a microorganism which is present in the gut or other organ of an animal in respect of which diagnosis is sought such as, for example, *E.coli*.

Conventional immunoassays can be used to perform this embodiment of the invention. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive

types, as well as the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target. It will be readily apparent to the skilled technician how to modify or optimise such assays to perform this embodiment of the present invention, and all such modifications and optimisations are encompassed by the present invention.

In one alternative embodiment, the present invention contemplates a method of identifying whether or not an animal has suffered from a past infection, or is currently infected with *L. intracellularis* or a related microorganism thereto, said method comprising contacting blood or serum derived from said animal with the immunogenic polypeptide of the invention for a time and under conditions sufficient for an antigen:antibody complex to form, and detecting said complex formation. This embodiment differs from the embodiment described *supra* in that it relies upon the detection of circulating antibodies against *L. intracellularis* or related organism in the animals blood or serum which are present as a consequence of a past or present infection by this pathogen. However, it will be apparent to those skilled in the art that the principle of the assay format is the same. As with other embodiments of the invention referred to *supra*, conventional immunoassays can be used. Persons skilled in the art will readily be capable of varying known immunoassay formats to perform the present embodiment. This embodiment of the invention can also utilise derivatives of blood and serum which comprise immunologically interactive molecules such as, for example, partially-purified IgG or IgM fractions and buffy coat samples, amongst others. The preparation of such fractions will also be known to those skilled in the art.

A further aspect of the present invention provides an isolated nucleic acid molecule which comprises a sequence of nucleotides that encodes, or is complementary to a nucleic acid molecule that encodes a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, including any and all genes selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes as defined hereinabove.

In a preferred embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a polypeptide that is immunologically cross-reactive with *L. intracellularis* or other causative agent of PPE, wherein said nucleotide sequence is selected from the group consisting of:

(i) a nucleotide sequence having at least about 60% sequence identity overall to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(ii) a nucleotide sequence having at least about 60% sequence identity overall to *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a nucleotide sequence which comprises at least about 15 contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17;

(iv) a nucleotide sequence which comprises at least about 15 contiguous nucleotides of *L. intracellularis* DNA contained within a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(v) a nucleotide sequence which hybridizes under at least low stringency conditions to a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, and 17 or a complementary nucleotide sequence thereto;

(vi) a nucleotide sequence which hybridizes under at least low stringency conditions to *L. intracellularis* DNA contained within a plasmid selected from

the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN); and

(vii) a homologue, analogue or derivative of any one of (i) to (vi) which encodes a polypeptide which mimics a B-cell or T-cell epitope of *Lawsonia spp.*

For the present purpose, a "homologue" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which encodes a polypeptide that is immunologically cross-reactive to a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, but which includes one or more nucleotide substitutions, insertions, deletions, or rearrangements.

An "analogue" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which encodes a polypeptide which is immunologically cross-reactive to a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, but which includes one or more non-nucleotide constituents not normally present in said isolated nucleic acid molecule, such as, for example, carbohydrates, radiochemicals including radio nucleotides, reporter molecules such as, but not limited to biotin, DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

A "derivative" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains at least about 60% nucleotide sequence identity to 15 or more contiguous nucleotides present in the nucleotide sequence of a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM,* and *ytfN* genes.

Generally, a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM,* or *ytfN* gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of the gene, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional nucleotide sequence variants are characterised by the removal of one or more nucleotides from the gene. Substitutional nucleotide sequence variants are those in which at least one nucleotide in the gene sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place. In a preferred embodiment, such substitutions are selected based on the degeneracy of the genetic code, as known in the art, with the resulting substitutional variant encoding the amino acid sequence of a flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, or ytfN polypeptide.

Preferred homologues, analogues and derivatives of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene comprise a sequence of nucleotides which has at least about 80%identity, even more preferably at least about 90% identity, and yet still more preferably at least about 95% identity to said gene.

In determining whether or not two nucleotide sequences fall within these percentage limits, reference is made to the description *supra* of methods for conducting a side-by-side comparison or multiple alignment of nucleotide sequences.

Alternatively or in addition, preferred homologues, analogues and derivatives of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene comprise a sequence of nucleotides which hybridizes under at least moderate stringency conditions and to the nucleotide sequence of said gene, or to a nucleic acid fragment comprising at least about 20 contiguous nucleotides in length derived therefrom, and even more preferably, under high stringency conditions to said gene, or to said nucleic acid

fragment. For the purposes of defining the level of stringency, reference is made to the description hereinabove of hybridization stringencies.

In a more preferred embodiment, such a nucleotide sequence encodes a polypeptide that is immunologically cross-reactive with *L. intracellularis* or other causative agent of PPE.

In a particularly preferred embodiment, the isolated nucleic acid molecule of the present invention comprises or consists of a nucleotide sequence selected from the group consisting of:

(i) a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1,3, 5, 7, 9, 11, 13, 15, and 17;

(ii) a nucleotide sequence of the *L. intracellularis* DNA contained within a deposited plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN);

(iii) a nucleotide sequence that encodes the same polypeptide as (i) or (ii), wherein said polypeptide is selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides; and

(iv) a nucleotide sequence that is complementary to (i) or (ii) or (iii).

The present invention clearly encompasses genetic constructs comprising the subject nucleic acid molecule in an expressible format suitable for the preparation of a recombinant immunogenic polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides, such as for use in recombinant univalent or polyvalent recombinant vaccines.

In such cases, the nucleic acid molecule will be operably connected to a promoter

sequence which can thereby regulate expression of said nucleic acid molecule in a prokaryotic or eukaryotic cell as described *supra*.

The genetic construct optionally further comprises a terminator sequence. The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. A "terminator" is a nucleotide sequence, generally located within the 3'-non-translated region of a gene or mRNA, comprising a polyadenylation signal to facilitate the post-transcriptional addition of a polyadenylate sequence to the 3'-end of a primary mRNA transcript. Terminator sequences may be isolated from the genetic sequences of bacteria, fungi, viruses, animals and/or plants. Terminators active in animal cells are known and described in the literature.

In a preferred embodiment, the genetic construct can be a cloning or expression vector, as known in the art, such as a plasmid, cosmid, or phage, comprising a nucleic acid molecule of the present invention, and host cells transformed or transfected therewith. In a non-limiting embodiment, the vector is a plasmid selected from the group consisting of AGAL Accession Nos: NM00/16476 (plasmid pGTE#1 glnH); NM00/16477 (plasmid pGTE#2 flhB); NM00/16478 (plasmid pGTE#3 fliR); NM00/16479 (plasmid pGTE#4 motA/B); NM00/16480 (plasmid pGTE#5 tlyC); NM00/16481 (plasmid pGTE#6 ntrC); NM00/16482 (plasmid pGTE#7 ytfM); and NM01/23286 (plasmid pGTE#8 ytfN).

The genetic constructs of the present invention are particularly useful for producing the immunogenic component of the vaccine composition described herein or for use in a DNA vaccine.

A range of genetic diagnostic assays to detect infection of an animal by *L. intracellularis* or a related microorganism can be employed using the nucleic acid molecule described herein such as, for example, assays based upon the polymerase chain reaction (PCR) and nucleic acid hybridisation. All such assays are

contemplated in the present invention.

Accordingly, a still further aspect of the invention provides a diagnostic method of detecting *L. intracellularis* or related microorganism in a biological sample derived from an animal subject, said method comprising the steps of hybridising one or more probes or primers derived from a nucleotide sequence of a *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* gene as defined hereinabove, or a homologue, analogue or derivative thereof, to a DNA or RNA molecule present in said sample and then detecting said hybridisation using a detection means.

As used herein, the term "probe" refers to a nucleic acid molecule which is capable of being used in the detection of a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes. Probes may comprise DNA (single-stranded or double-stranded) or RNA (i.e., riboprobes) or analogues thereof.

The term "primer" refers to a probe as hereinbefore defined which is further capable of being used to amplify a nucleotide sequence from *L. intracellularis* or a related microorganism thereto in a PCR.

Preferred probes and primers include fragments of a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes, including synthetic single-stranded DNA or RNA molecules of at least about 15 nucleotides in length.

Preferably, probes and primers according to this embodiment will comprise at least about 20 contiguous nucleotides in length from a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes, even more preferably at least about 25 contiguous nucleotides, still even more preferably at least about 50 contiguous nucleotides, and even more preferably at least about 100 nucleotides to about 500 nucleotides in length from said gene. Probes and primers comprising the full-length gene or a complementary nucleotide sequence thereto are

also encompassed by the present invention.

Probes or primers can comprise inosine, adenine, guanine, thymidine, cytidine or uracil residues or functional analogues or derivatives thereof that are capable of being incorporated into a polynucleotide molecule, provided that the resulting probe or primer is capable of hybridising under at least low stringency conditions to a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes, or is at least about 60% identical to one strand of said gene.

The biological sample according to this aspect of the invention includes any organ, tissue, cell or exudate which contains or is likely to contain *L. intracellularis* or a nucleic acid derived therefrom. A biological sample can be prepared in a suitable solution such as, for example, an extraction buffer or suspension buffer. The present invention extends to the testing of biological solutions thus prepared, the only requirement being that said solution at least comprises a biological sample as described herein.

The diagnostic assay of the present invention is useful for the detection of *L. intracellularis* or a microorganism which is related thereto which expresses a polypeptide selected from the group consisting of flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM, and ytfN polypeptides.

The present invention clearly contemplates diagnostic assays which are capable of both genus-specific and species-specific detection. Accordingly, in one embodiment, the probe or primer, or a homologue, analogue or derivative thereof, comprises DNA capable of being used to detect multiple *Lawsonia spp.* In an alternative embodiment, the probe or primer or a homologue, analogue or derivative thereof comprises DNA capable of being used to distinguish *L. intracellularis* from related microorganisms.

Less-highly conserved regions within the *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, or *ytfN* genes are particularly useful as species-specific probes and/or primers for the

detection of *L. intracellularis* and very closely related species.

Furthermore, the diagnostic assays described herein can be adapted to a genus-specific or species-specific assay by varying the stringency of the hybridisation step. Accordingly, a low stringency hybridisation can be used to detect several different species of *Lawsonia* in one or more biological samples being assayed, while a high stringency hybridisation can be used to distinguish *L. intracellularis* from such other species.

The detection means according to this aspect of the invention may be any nucleic acid-based detection means such as, for example, nucleic acid hybridisation techniques or paper chromatography hybridisation assay (PACHA), or an amplification reaction such as PCR, or nucleic acid sequence-based amplification (NASBA) system. The invention further encompasses the use of different assay formats of said nucleic acid-based detection means, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), single-strand chain polymorphism (SSCP), amplification and mismatch detection (AMD), interspersed repetitive sequence polymerase chain reaction (IRS-PCR), inverse polymerase chain reaction (iPCR), *in situ* polymerase chain reaction and reverse transcription polymerase chain reaction (RT-PCR), amongst others.

Where the detection means is a nucleic acid hybridisation technique, the probe can be labelled with a reporter molecule capable of producing an identifiable signal (e.g., a radioisotope such as ^{32}P or ^{35}S, or a biotinylated molecule). According to this embodiment, those skilled in the art will be aware that the detection of said reporter molecule provides for identification of the probe and that, following the hybridisation reaction, the detection of the corresponding nucleotide sequences in the biological sample is facilitated. Additional probes can be used to confirm the assay results obtained using a single probe.

A variation of the nucleic acid hybridisation technique contemplated by the present

invention is the paper chromatography hybridisation assay (PACHA) described by Reinhartz *et al.* (1993) and equivalents thereof, wherein a target nucleic acid molecule is labelled with a reporter molecule such as biotin, applied to one end of a nitrocellulose or nylon membrane filter strip and subjected to chromatography under the action of capillary or other forces (e.g., an electric field) for a time and under conditions sufficient to promote migration of said target nucleic acid along the length of said membrane to a zone at which a DNA probe is immobilised thereto such as, for example, in the middle region. According to this detection format, labelled target nucleic acid comprising the *Lawsonia spp.* nucleotide sequences complementary to the probe will hybridise thereto and become immobilised in that region of the membrane to which the probe is bound. Non-complementary sequences to the probe will diffuse past the site at which the probe is bound. The target nucleic acid may comprise a crude or partially-pure extract of DNA or RNA or, alternatively, an amplified or purified DNA. Additional variations of this detection means which utilise the nucleotide sequences described herein are clearly encompassed by the present invention.

Wherein the detection means is a RFLP, nucleic acid derived from the biological sample, in particular DNA, is digested with one or more restriction endonuclease enzymes and the digested DNA is subjected to electrophoresis, transferred to a solid support such as, for example, a nylon or nitrocellulose membrane, and hybridised to a probe optionally labelled with a reporter molecule as hereinbefore defined. According to this embodiment, a specific pattern of DNA fragments is displayed on the support, wherein said pattern is preferably specific for a particular *Lawsonia spp.*, to enable the user to distinguish between different species of the bacterium.

Wherein the detection means is an amplification reaction such as, for example, a polymerase chain reaction or a nucleic acid sequence-based amplification (NASBA) system or a variant thereof, one or more nucleic acid primer molecules of at least 15 contiguous nucleotides in length derivable from a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes is

hybridised to nucleic acid derived from a biological sample, and nucleic acid copies of the FlgE-encoding genetic sequences in said sample, or a part or fragment thereof, are enzymically-amplified.

Those skilled in the art will be aware that there must be a sufficiently high percentage of nucleotide sequence identity between the primers and the sequences in the biological sample template molecule to which they hybridise (i.e., the "template molecule"). As stated previously, the stringency conditions can be selected to promote hybridisation.

Preferably, each primer is at least about 95% identical to a region of a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes in the template molecule to which it hybridises.

Those skilled in the art will also be aware that, in one format, PCR provides for the hybridisation of non-complementary primers to different strands of the template molecule, such that the hybridised primers are positioned to facilitate the 5′→ 3′ synthesis of nucleic acid in the intervening region, under the control of a thermostable DNA polymerase enzyme. As a consequence, PCR provides an advantage over other detection means in so far as the nucleotide sequence in the region between the hybridised primers may be unknown and unrelated to any known nucleotide sequence.

In an alternative embodiment, wherein the detection means is AFLP, the primers are selected such that, when nucleic acid derived from the biological sample, in particular DNA, is amplified, different length amplification products are produced from different *Lawsonia spp.* The amplification products can be subjected to electrophoresis, transferred to a solid support such as, for example, a nylon or nitrocellulose membrane, and hybridised to a probe optionally labelled with a reporter molecule as hereinbefore described. According to this embodiment, a specific pattern of amplified DNA fragments is displayed on the support, said pattern optionally *specific for a*

particular *Lawsonia ssp.,* to enable the user to distinguish between different species of the bacterium in much the same way as for RFLP analysis.

The technique of AMD facilitates, not only the detection of *Lawsonia spp.* DNA in a biological sample, but also the determination of nucleotide sequence variants which differ from the primers and probes used in the assay format. Wherein the detection means is AMD, the probe is end-labelled with a suitable reporter molecule and mixed with an excess of the amplified template molecule. The mixtures are subsequently denatured and allowed to renature to form nucleic acid "probe:template hybrid molecules" or "hybrids", such that any nucleotide sequence variation between the probe and the temple molecule to which it is hybridised will disrupt base-pairing in the hybrids. These regions of mismatch are sensitive to specific chemical modification using hydroxylamine (mismatched cytosine residues) or osmium tetroxide (mismatched thymidine residues), allowing subsequent cleavage of the modified site using piperidine. The cleaved nucleic acid may be analysed using denaturing polyacrylamide gel electrophoresis, followed by standard nucleic acid hybridisation as described *supra,* to detect the *Lawsonia*-derived nucleotide sequences. Those skilled in the art will be aware of the means of end-labelling a genetic probe according to the performance of the invention described in this embodiment.

According to this embodiment, the use of a single end-labelled probe allows unequivocal localisation of the sequence variation. The distance between the point(s) of sequence variation and the end-label is represented by the size of the cleavage product.

In an alternative embodiment of AMD, the probe is labelled at both ends with a reporter molecule, to facilitate the simultaneous analysis of both DNA strands.

Wherein the detection means is RT-PCR, the nucleic acid sample comprises an RNA molecule which is a transcription product of *Lawsonia*-derived DNA or a homologue, analogue or derivative thereof. As a consequence, this assay format is particularly

useful when it is desirable to determine expression of one or more *Lawsonia* genes. According to this embodiment, the RNA sample is reverse-transcribed to produce the complementary single-stranded DNA which is subsequently amplified using standard procedures.

Variations of the embodiments described herein are described in detail by McPherson *et al.* (1991).

The present invention clearly extends to the use of any and all detection means referred to *supra* for the purposes of diagnosing *Lawsonia spp.* and in particular *L. intracellularis* infection in animals.

The amplification reaction detection means described *supra* can be further coupled to a classical hybridisation reaction detection means to further enhance sensitivity and specificity of the inventive method, such as by hybridising the amplified DNA with a probe which is different from any of the primers used in the amplification reaction.

Similarly, the hybridisation reaction detection means described *supra* can be further coupled to a second hybridisation step employing a probe which is different from the probe used in the first hybridisation reaction.

A further aspect of the invention provides an isolated probe or primer derived from a gene selected from the group consisting of *flhB, fliR, ntrC, glnH, motA, motB, tlyC, ytfM*, and *ytfN* genes. Preferably, the probe or primer of the invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 19 to SEQ ID NO: 68 or a complementary nucleotide sequence thereto.

The present invention does not extend to any nucleic acid or polypeptide of *Camplylobacter* or *Helicobacter* that was disclosed publicly before the filing date or priority date of this application, or otherwise takes priority over the instant application, and which is homologous to a nucleotide sequence or amino acid sequence of

Lawsonia spp. disclosed herein.

The present invention is further described with reference to the following non-limiting Examples.

EXAMPLE 1

Molecular Cloning of *Lawsonia intracellularis* genes

Isolation of DNA and construction of DNA libraries

L. intracellularis DNA was purified from pig intestinal mucosa isolated from the ileum of pigs experimentally infected with *L. intracellularis*. DNA purification from homogenized intestinal mucosa was performed according to the method of Nollau *et al.* (1996); or alternatively, by phenol extraction and sodium acetate-ethanol precipitation of DNA.

To facilitate cloning of *L. intracellularis* gene sequences, several genomic libraries were constructed. These libraries were specifically modified by ligation of a known sequence (Vectorette II™, Genosys Biotechnologies, Inc., The Woodlands, TX) to the 5'- and 3'- ends of restricted DNA fragments. Vectorette™ libraries were constructed by separately digesting aliquots of *L. intracellularis*-infected pig mucosal DNA extract with restriction endonucleases *Hin*dIII, *Eco*RI, *Dra*I or *Hpa*I at 37°C overnight. The reaction was then spiked with additional fresh restriction enzyme and adjusted to 2 mM ATP, 2 mM DTT final concentration. Vectorette™ tailing was carried out by addition of T_4 DNA Ligase (1 unit) plus 3 pMol of the appropriate compatible Vectorette™ linker (*Hin*dIII Vectorette™: *Hin*dIII-digested DNA; *Eco*RI: *Eco*RI digested DNA; Blunt: *Dra*I-, *Hpa*I- digested DNA). The mixture was incubated for three cycles, each cycle consisting of 20°C for 60 min; followed by 37°C for 30 min, to complete the tailing reaction. Reaction volumes were then adjusted to 200 µl with water, and reactions were stored at -20°C.

EXAMPLE 2

Expression of the *YtfN and YtfM* genes of *L. intracellularis*

i) Isolation of a C-terminal fragment of ytfN gene by genome walking

The complete sequence of the *L. intracellularis YtfN* gene was determined from genomic DNA and is set forth herein as SEQ ID NO: 17. Based upon the 2,035 bp sequence obtained for the amino terminal portion of the *ytfN* gene fragment, oligonucleotide primer KWK-Li-YtfN-4C (SEQ ID NO:29) was designed and synthesized (Life Technologies; Rockville, MD). This oligonucleotide binds within the 3'-region of the *YtfN* gene in the *L. intracellularis* chromosome to allow amplification of DNA downstream of the existing gene fragment. For polymerase chain amplification, primer KWK-Li-YtfN-4C (SEQ ID NO:29) was used in combination with a Vectorette™ specific oligonucleotide primer (ER70; SEQ ID NO:108) in 50 µl reactions containing 1x PCR Buffer II (Perkin Elmer; Foster City, CA), 2.0 mM $MgCl_2$, 250 µM each deoxy-NTP, 50 pMol each primer, and 2.5 U AmpliTaq™ Gold (Perkin Elmer) thermostable polymerase. Reactions were performed with 1 µl of the Vectorette™ libraries as DNA template. Amplification was carried out as follows: denaturation (94°C, 9 min); 40 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and polymerization (72°C, 4.0 min); this was followed by a final extension at 72°C for 7 minutes.

The amplified products were visualized by separation on a 1.0% agarose gel (Sigma; St. Louis, Mo). Screening of the *HpaI* library by PCR resulted in amplification of a fragment approximately 1.5 kb in length. The PCR product was purified using a QIAquick™ PCR Purification kit (Qiagen; Valencia, CA) and cloned into the TA cloning site of pCR2.1-TOPO (Invitrogen; Carlsbad, CA); the ligated product was transformed into Max Efficiency *E. coli* DH5α cells (Life Technologies; Rockville, MD). Sequence analysis of the cloned fragment failed to identify a termination codon for *ytfN*. Therefore, oligonucleotide primer KWK-Li-YtfN-12C (SEQ ID NO:21) was designed and synthesized to be used in a second round of PCR amplification using the Vectorette™ libraries. The above-mentioned conditions for amplification were used, and products were visualized by agarose gel electrophoresis. A fragment

approximately 1.6 kb in length was amplified from the *DraI* library. The PCR product was purified using a QIAquick PCR Purification kit, cloned into pCR2.1-TOPO, and subsequently transformed into Max Efficiency *E. coli* DH5α cells. Sequence analysis of the fragment identified a terminal TAA codon indicating the end of the *ytfN* gene.

ii) Determination of genomic sequence of complete ytfN gene

Results from the preliminary sequencing described above were used to design oligonucleotide primers for the specific amplification of two overlapping *ytfN* gene fragments directly from *L. intracellularis* chromosomal DNA. These products encompass the entire *ytfN* gene and were sequenced directly in an attempt to avoid introduction of sequence artifacts due to mutations which might arise during PCR amplification and subsequent cloning steps. To obtain the first of the two fragments, PCR amplifications were carried out in triplicate and contained 100 pMol of primers YtfN-D (SEQ. ID NO:45) and YtfN-U (SEQ. ID NO:46), 100 ng purified chromosomal DNA, 1x PC2 buffer (Ab Peptides; St. Louis, MO), 200 μM each dNTP, 15 U Klen*Taq*1 (Ab Peptides) and 0.3 U cloned *Pfu* (Stratagene; La Jolla, CA) thermostable polymerases in a 100 μl final sample volume. Conditions for amplification consisted of denaturation (94°C, 9 min), followed by 40 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and polymerization (72°C, 4.0 min), and a final extension at 72°C for 7 min. To obtain the second (overlapping) fragment, PCR amplifications were carried out in triplicate as described above, except that primers KWK-Li-YtfN-12C (SEQ ID NO:21) and KWK-LI-YtfN-15N (SEQ ID NO:24) were used. Conditions for amplification consisted of denaturation (94°C, 9 min), followed by 40 cycles of denaturation (94°C, 30 sec), annealing (55°C, 30 sec), and polymerization (72°C, 2.5 min), and a final extension at 72°C for 7 min.

Following amplification, each set of triplicate samples was pooled and the specific product from each was purified (QIAquick™ PCR Purification kit). Both purified DNA fragments were then subjected to direct sequence analysis using DyeDeoxy termination reactions on an ABI automated DNA sequencer (Lark Technologies Inc., Houston, TX).

Synthetic oligonucleotide primers (SEQ ID NOs:21, 24, 26-38, 43-46, 51-53, and 55-60) were used to sequence both DNA strands of the amplified products.

The *ytfN* ORF extends from nucleotides 1-4149 of SEQ ID NO:17 and encodes a 1382 amino acid protein (SEQ ID NO:18), having a theoretical molecular weight of 150,887 Daltons. The sequence of the amino terminus of the encoded protein resembles a prokaryotic signal sequence (von Heijne, 1985; Nielsen, *et al.*, 1997), although the precise site of cleavage is not presently known. The *ytfN* ORF was compared against existing nucleotide and protein databases using the Basic Local Alignment Search Tool (BLAST) programs (Altschul, *et al.*, 1990). The entry with which it shared the greatest homology was a hypothetical 40.5 kDa protein from *Zymomonas mobilis*. The second-most-significant homologous sequence identified was a YtfN homolog from *Neisseria meningitidis*.

iii) Cloning of recombinant ytfM gene into expression vectors

For the purpose of recombinant protein expression, both the *ytfM* and *ytfN* genes or fragments thereof were cloned without the sequences encoding their respective signal peptides.

The *ytfM* gene was amplified from *L. intracellularis* chromosomal DNA using oligonucleotide primers RA202-b (SEQ ID NO: 50) and RA201-b (SEQ ID NO: 49). For polymerase chain amplification, triplicate 50 µl reactions were set up with eachcontaining 100 ng of chromosomal DNA as template, 1x PC2 buffer, 200 µM each dNTP, 50 pMol each primer, 7.5 U Klen*Taq*1 and 0.15 U cloned *Pfu* thermostable polymerases. Amplification was carried out as follows: denaturation (94°C, 9 min); 40 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and polymerization (72°C, 2.5 min), followed by a final extension at 72°C for 7 minutes. Following amplification, the samples were purified (QIAquick™ PCR Purification kit) and pooled. The purified PCR product was cloned directly into the TA cloning site of both pBAD-TOPO and pBAD/Thio-TOPO (Invitrogen). The ligated

products were transformed into Max Efficiency *E. coli* DH5α cells. The predicted amino terminal sequence of the encoded protein expressed from pBAD-TOPO:YtfM would consist of the vector-encoded sequence MGSGSGDDDDKLALLTM (SEQ ID NO: 61) followed immediately by the sequence ATSITTS (SEQ ID NO: 62) beginning at Alanine-24 of the YtfM ORF (SEQ ID NO:16). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit (Qiagen). This plasmid was transformed into *E. coli* BL21 (Novagen; Madison, WI) and BL21-CodonPlus-RIL cells (Stratagene); a clone was identified in each strain that contained the appropriate plasmid.

The predicted amino terminal sequence of the encoded fusion protein expressed from pBAD/Thio-TOPO:YtfM would consist of the thioredoxin protein and a 15 amino acid residue linker followed immediately by the sequence ATSITTS (SEQ ID NO: 62) beginning at Alanine-24 of the YtfM ORF (SEQ ID NO:16). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21 and BL21-CodonPlus-RIL cells; a clone was identified in each strain that contained the appropriate plasmid.

For cloning into pET-30a, the purified PCR product encoding YtfM was digested with *Bam*HI and *Nco*I, then purified using a QIAquick™ PCR Purification kit. pET-30a was also digested with *Bam*HI and *Nco*I; the linearized plasmid was purified using a JETsorb™ kit (Genomed; Frederick, MD) prior to ligation. The ligated product was transformed into Max Efficiency *E. coli* DH5α cells. The predicted amino terminal sequence of the encoded fusion protein expressed from pET-30a:YtfM would consist of MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAM (SEQ ID NO: 63) encoded by the vector, followed by the sequence ATSITTS (SEQ ID NO: 62) beginning at Alanine-24 of the YtfM ORF (SEQ ID NO:16). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed

into *E. coli* BL21(DE3) (Novagen) and BL21-CodonPlus(DE3)-RIL cells (Stratagene); a clone was identified in each strain that contained the appropriate plasmid.

The *ytfM* gene was also amplified from *L. intracellularis* chromosomal DNA by PCR amplification using oligonucleotide primers RA200 (SEQ ID NO: 47) and RA201 (SEQ ID NO: 48). Duplicate 50 µl reactions were set up each containing 100 ng of chromosomal DNA as template, 1x PC2 buffer, 200 µM each dNTP, 50 pMol each primer, 7.5 U Klen*Taq*1 and 0.15 U cloned *Pfu* thermostable polymerases. Amplification was carried out as follows: denaturation (94°C, 9 min); 30 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and polymerization (72°C, 2 min), followed by a final extension at 72°C, for 7 minutes. Following amplification, the samples were purified (QIAquick™ PCR Purification kit) and pooled. The purified PCR product was cloned directly into the TA cloning site of pCR2.1-TOPO. The ligated product was transformed into Max Efficiency *E. coli* DH5α cells. A clone containing the appropriate plasmid was identified, propagated, and plasmid DNA was isolated using a QIAprep Spin Miniprep kit. Following digestion of the plasmid with *Eco*RI, a fragment corresponding to bp 437 of SEQ ID NO:15 to the *Eco*RI site in the MCS of pCR2.1-TOPO was purified using a JETsorb™ kit. pET-30a was also digested with *Eco*RI, and purified using a QIAquick PCR™ Purification kit. The two fragments were ligated and transformed into Max Efficiency *E. coli* DH5a cells. The predicted amino terminal sequence of the encoded fusion protein would consist of MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGS (SEQ ID NO: 64) encoded by the vector followed by the sequence EFNLSKG (SEQ ID NO: 65) beginning at Aspartate-146 of the YtfM ORF (SEQ ID NO:16). A clone containing the plasmid with the gene fragment inserted in the proper orientation was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL cells; a clone was identified that contained the appropriate plasmid.

iv) Cloning of recombinant ytfN gene into expression vectors

The 5' half of the *ytfN* gene, excluding that encoding the signal sequence, was amplified from *L. intracellularis* chromosomal DNA using oligonucleotide primers RA205-b (SEQ ID NO: 53) and RA204-b (SEQ ID NO: 52). For polymerase chain amplification, triplicate 100 µl reactions were set up each containing 100 ng of chromosomal DNA as template, 1x PC2 buffer, 200 µM each dNTP, 100 pMol each primer, 15 U KlenTaq1 and 0.3 U cloned *Pfu* thermostable polymerases. Amplification was carried out as follows: denaturation (94°C, 9 min); 40 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and polymerization (72°C, 4 min), followed by a final extension at 72°C for 7 minutes. Following amplification, the samples were purified (QIAquick™ PCR Purification kit) and pooled. The purified PCR product was cloned directly into the TA cloning site of both pBAD-TOPO and pBAD/Thio-TOPO (Invitrogen). The ligated products were transformed into Max Efficiency *E. coli* DH5α cells. The predicted amino terminal sequence of the encoded protein expressed from pBAD-TOPO:YtfN would consist of the vector-encoded sequence MGSGSGDDDDKLALGHM (SEQ ID NO: 66) followed immediately by the sequence RTSTGIA (SEQ ID NO: 67) beginning at Arginine-33 of the YtfN ORF (SEQ ID NO:18). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21-CodonPlus-RIL cells; a clone was identified that contained the appropriate plasmid.

The predicted amino terminal sequence of the encoded protein expressed from pBAD/Thio-TOPO:YtfN would consist of the thioredoxin protein and a 15 amino acid linker followed immediately by the sequence RTSTGIA (SEQ ID NO: 67) beginning at Arginine-33 of the YtfN ORF (SEQ ID NO:18). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21-CodonPlus-RIL cells; a clone was identified that contained the appropriate plasmid.

For cloning into pET-30a, the purified PCR product was digested with *Bam*HI and *Nde*I and extracted using a QIAquick PCR Purification kit. The linearized plasmid was purified using a JETsorb™ kit prior to ligation. The ligated product was transformed into Max Efficiency *E. coli* DH5α cells. The predicted amino terminal sequence of the protein expressed from pET-30a:YtfN would consist of Met encoded by RA205-b (SEQ ID NO: 53) followed by the sequence RTSTGIA (SEQ ID NO: 67) beginning at Arginine-33 of the YtfN ORF (SEQ ID NO:18). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL and BL21-CodonPlus(DE3)-RP cells (Stratagene); a clone was identified in each strain that contained the appropriate plasmid.

Utilizing oligonucleotide primers RA205-b (SEQ ID NO: 53) and KWK-Li-YtfN-BglII-3' (SEQ ID NO:39), an approximate 1kb fragment encoding the N-terminal portion of *YtfN*, excluding the signal sequence, was amplified by PCR. Triplicate 50 µl reactions were set up each containing 100 ng of chromosomal DNA as template, 1x PC2 buffer, 200 µM each dNTP, 50 pMol each primer, 7.5 U Klen*Taq*1 and 0.15 U cloned *Pfu* thermostable polymerases. Amplification was carried out as follows: denaturation (94°C, 9 min); 40 cycles of denaturation (94°C, 30 sec), annealing (55°C, 30 sec), and polymerization (72°C, 1 min); followed by a final extension at 72°C for 7 minutes. Following amplification, the samples were purified (QIAquick™ PCR Purification kit) and pooled. The purified PCR product was cloned directly into the TA cloning site of both pBAD-TOPO and pBAD/Thio-TOPO. The ligated products were transformed into Max Efficiency *E. coli* DH5α cells. The predicted amino terminal sequence of the protein expressed from pBAD-TOPO would consist of the vector-encoded sequence MGSGSGDDDDKLALGHM (SEQ ID NO: 66) followed immediately by the sequence RTSTGIA (SEQ ID NO: 67) beginning at Arginine-33 of the YtfN ORF (SEQ ID NO:18); the protein would terminate with Isoleucine-332 of *ytfN* (SEQ ID NO:18). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep

Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21-CodonPlus-RIL cells; a clone was identified that contained the appropriate plasmid.

The predicted amino terminal sequence of the encoded fusion protein expressed from pBAD/Thio-TOPO:YtfN would consist of the thioredoxin protein and a 15 amino acid linker followed immediately by the sequence RTSTGIA (SEQ ID NO: 67) beginning at Arginine-33 of the YtfN ORF (SEQ ID NO:18); again, the polypeptide would terminate with Isoleucine-332 of *ytfN* (SEQ ID NO:18). A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit. This plasmid was transformed into *E. coli* BL21-CodonPlus-RIL cells; a clone was identified that contained the appropriate plasmid.

In order to generate a construct consisting of the above-described 1 kb (5' to *Bgl*II site) *ytfN* fragment in pET30a, the plasmid pET-30a:YtfN containing the 5' half of *ytfN* amplified using RA205-b (SEQ ID NO: 53) and RA204-b (SEQ ID NO: 52) was digested with *Bgl*II and *Bam*HI, thus excising the gene sequence downstream of the *Bgl*II site within *ytfN*. The fragment containing the vector and *ytfN* sequence up to the *Bgl*II site was purified using a JETsorb™ kit, and the remaining fragment was religated and transformed into *E. coli* Max Efficiency DH5α cells. The predicted amino terminal sequence of the protein expressed would consist of Met encoded by RA205-b (SEQ ID NO: 53) followed by the sequence RTSTGIA (SEQ ID NO: 67) beginning at Arginine-33 of the YtfN ORF (SEQ ID NO:18). The polypeptide would terminate with Isoleucine-332 of SEQ ID NO:18, followed by a C-terminal extension consisting of DPNSSSVDKLAAALEHHHHHH (SEQ ID NO: 68) encoded by the vector. A clone containing the appropriate plasmid was identified, and purified plasmid was isolated from a small-scale broth culture using a QIAprep Spin Miniprep kit (Qiagen). This plasmid was transformed into *E. coli* BL21(DE3) and BL21-CodonPlus(DE3)-RIL cells; a clone was identified in each strain that contained the appropriate plasmid. Stocks of the clone containing this plasmid in *E. coli* BL21 (DE3) were frozen at -80°C.

v) Expression of Recombinant YtfN Polypeptide

Frozen working stock of the *E. coli* BL21 (DE3) transformant harboring pET-30a containing the 5' portion of *ytfN* up to the *Bgl*II site (eg. corresponding to amino acids 33-332 of the encoded YtfN protein) was thawed and seeded at a 1:5000 dilution in RWLDM/G vi defined medium [K_2HPO_4 (6 g/L), KH_2PO_4 (3 g/L), $(NH_4)_2SO_4$ (5 g/L), NaCl (2 g/L), 0.2 mL $CaCl_2$ (15 g/L), 0.4 mL $FeCl_3.6H_2O$ (5 g/L), 0.4 ml $MgSO_4.7H_2O$ (480 g/L), $ZnCl_2$ (6.5 g/L), $MnSO_4.H_2O$ (12 g/L), $Na_2MoO_4.2H_2O$ (5 g/L), $CuSO_4$ (1.5 g/L), $CoCl_2.6H_2O$ (2 g/L), H_3BO_3 (0.5 g/L), and 37% HCl (5ml/L)]. Kanamycin was also added to a concentration of 25 μg/ml to maintain the expression plasmid. The culture was grown under fed-batch (50% glycerol) in a 5 liter working volume BioFlow 3000 fermentor (New Brunswick Scientific; Edison, NJ) at 37°C until A_{625} was 2.9. At this time, IPTG was added to 0.1 mM, and culture samples were collected at 0, 2.25, and 3 hours post induction to monitor expression of recombinant YtfN (see Figure). The primary culture was maintained 28 hours post inoculation then immediately chilled, and wet cells were collected by centrifugation and stored at -20°C. Data showing expression of ytfN protein are presented in Figure 1.

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